医学生物学 SCI 论文经典句子汇编

2009 年 2 月通知：

目前所有的内容均已上网，可以在线免费查询，信息量多达 1000 多页！

更名《**SCI 论文写作宝典**》，链接：http://www.medsci.cn/query.asp **免费提供，需先注册！**

说明：

1 http://www.medsci.cn 编写，仅供编辑参考使用，写作时个别可以根据单词可章节查找!

2 内容均由工作人员细心整理而成，均取自一线 SCI 杂志的上千篇文献，进行了总结，在写作时，读一遍本文，将获益匪浅！

3 如果需要 SCI 论文的翻译，修改，以及英语母语化润色，可以访问我们的网站 www.medsci.cn ，另外我们陆续将优秀的整理好的内容也将放在我们的网站上，欢迎免费下载和传播！我们相信您的个人品德，一定会尊重我们的汗水，反对盗版或消去我们的版权，谢谢！

Title

要求简练，精确，突出文章关键词

- Compassionate use of bevacizumab (Avastin) in children and young adults with refractory or recurrent solid tumors.
- Bevacizumab-induced transient remodeling of the vasculature in neuroblastoma xenografts results in improved delivery and efficacy of systemically administered chemotherapy.
- Proteomics Approaches to the Systems Biology of Cardiovascular Diseases
- Pre- and post-natal treatment of hemophagocytic lymphohistiocytosis.
- Lack of early bevacizumab-related skeletal radiographic changes in children with neuroblastoma.
- Interleukin-4 activates androgen receptor through CBP/p300
- Trisomy 8 in an allogeneic stem cell transplant recipient representative of a donor-derived constitutional abnormality.
- Disruption of diacylglycerol metabolism impairs the induction of T cell anergy
- T cell anergy is reversed by active Ras and is regulated by diacylglycerol kinase-
- High-dose conformal RT improves tumor control in patients with prostate cancer
- Vitamin D concentration does not affect the risk of prostate cancer
- Liver resection with salvage transplantation for hepatocellular carcinoma
- The impact of histopathologic diagnosis on the proper management of testis neoplasms
- Prostate stem cell antigen is associated with diffuse-type gastric cancer
- Multiple myeloma: high-risk immunophenotypes identified
- Increased c-kit expression predicts poor outcome in acute myeloid leukemia
- Global Analysis of the Meiotic Crossover Landscape
- Serum Response Factor Is Required for Sprouting Angiogenesis and Vascular Integrity
- Integrin Trafficking Regulated by Rab21 Is Necessary for Cytokinesis
- Reduced Translocation of Nascent Prion Protein During ER Stress Contributes to Neurodegeneration

- Effects of oral niacin on endothelial dysfunction in patients with coronary artery disease: Results of the randomized, double-blind, placebo-controlled INEF study.
- Global experiences with vardenafil in men with erectile dysfunction and underlying conditions.
- Noninvasive cardiac imaging: implications for risk assessment in adolescents and young adults.
- Transforming growth factor beta1 T29C gene polymorphism and hypertension: Relationship with cardiovascular and renal damage.
- A comparison of hormone therapies on the urinary excretion of prostacyclin and thromboxane A2.
- Repair of an infected aortic aneurysm using an aortic allograft and a venous autograft: Report of a case.
- Circulating Leptin and Stress-induced Cardiovascular Activity in Humans.
- Effects of aspirin dose on ischaemic events and bleeding after percutaneous coronary intervention: insights from the PCI-CURE study.
- Long-term cardiovascular outcomes following ischemic heart disease in patients with and without peripheral vascular disease.
- Reduced renal function and sleep-disordered breathing in community-dwelling elderly men.
- Intracoronary pharmacotherapy in the management of coronary microvascular dysfunction.
- Inhibition of platelet aggregation by combined therapy with aspirin and cilostazol after off-pump coronary artery bypass surgery.
- Inhibition of CCR2 Ameliorates Insulin Resistance and Hepatic Steatosis in db/db Mice

几种特殊题目的写法：

（1）带：的写法，常在综述，病例报道，多中心研究试验中出现，不过现在在一般的 article 中也经常出现。如：

Slide into action: dynamic shuttling of HIV reverse transcriptase on nucleic acid substrates

Antiphospholipid syndrome presenting as unilateral renal artery occlusion: case report and literature review.

Volar Melanotic Macules in a Gardener: A Case Report and Review of the Literature.

The influence of sex on the chondrogenic potential of muscle-derived stem cells: Implications for cartilage regeneration and repair.

Advanced mast cell disease: an Italian Hematological Multicenter experience

Abstract 要求简洁，连贯

- The acquisition of metastatic ability by tumor cells is considered a late event in the evolution of malignant tumors. We report that untransformed mouse mammary cells that have been engineered to express the inducible oncogenic transgenes MYC and $Kras^{D12}$, or polyoma middle T, and introduced into the systemic circulation of a mouse can bypass transformation at the primary site and develop into metastatic pulmonary lesions upon

immediate or delayed oncogene induction. Therefore, previously untransformed mammary cells may establish residence in the lung once they have entered the bloodstream and may assume malignant growth upon oncogene activation. Mammary cells lacking oncogenic transgenes displayed a similar capacity for long-term residence in the lungs but did not form ectopic tumors.

- Almost two decades after *CFTR* was identified as the gene responsible for cystic fibrosis (CF), we still lack answers to many questions about the pathogenesis of the disease, and it remains incurable. Mice with a disrupted *CFTR* gene have greatly facilitated CF studies, but the mutant mice do not develop the characteristic manifestations of human CF, including abnormalities of the pancreas, lung, intestine, liver, and other organs. Because pigs share many anatomical and physiological features with humans, we generated pigs with a targeted disruption of both *CFTR* alleles. Newborn pigs lacking CFTR exhibited defective chloride transport and developed meconium ileus, exocrine pancreatic destruction, and focal biliary cirrhosis, replicating abnormalities seen in newborn humans with CF. The pig model may provide opportunities to address persistent questions about CF pathogenesis and accelerate discovery of strategies for prevention and treatment.
- Variable lymphocyte receptors (VLRs) rather than antibodies play the primary role in recognition of antigens in the adaptive immune system of jawless vertebrates. Combinatorial assembly of leucine-rich repeat (LRR) gene segments achieves the required repertoire for antigen recognition. We have determined a crystal structure for a VLR-antigen complex, VLR RBC36 in complex with the H-antigen trisaccharide from human blood type O erythrocytes, at 1.67 angstrom resolution. RBC36 binds the H-trisaccharide on the concave surface of the LRR modules of the solenoid structure where three key hydrophilic residues, multiple van der Waals interactions, and the highly variable insert of the carboxyl-terminal LRR module determine antigen recognition and specificity. The concave surface assembled from the most highly variable regions of the LRRs, along with diversity in the sequence and length of the highly variable insert, can account for the recognition of diverse antigens by VLRs.
- A 51-year-old man with a diagnosis of myelodysplasia and non-Hodgkin's lymphoma underwent an unmatched allogenic bone marrow transplantation and was treated posttransplant with chronic immunosuppressive medication. Eight months following transplantation, he presented with progressive dysarthria, cognitive and visual decline. Evaluation included brain magnetic resonance (MR) imaging demonstrating multifocal areas of increased T2 and FLAIR (fluid attenuated inversion recovery) signals involving the left frontal, parietal, and occipital lobes. The MR lesions demonstrated diffuse increased signal on DWI (diffusion-weighted images) and normal to low signal on ADC (apparent diffusion coefficients). Contrast-enhanced T1 images were unremarkable. Lumbar puncture revealed a mild elevation in cerebrospinal fluid (CSF) protein. CSF PCR assay for viral DNA fragments were negative on two occasions. Serum serology for HIV was negative as well. A brain biopsy was subsequently performed. The clinical and neuroimaging differential diagnoses as well as neuropathologic correlation are presented.
- In vitro-generated mesenchymal stem cells (MSCs) initially attracted interest for their ability to undergo differentiation toward cells of different lineages.

- These results suggested that
- However, there are still obstacles in
- The major challenge for successful drug development is identifying delivery strategies that can be translated to the clinic.
- This review will discuss progress in developing and testing small RNAi-based drugs and potential obstacles.
- This review highlights what
- In addition, there are indications that
- Proper consideration of all of these issues will be necessary in
- These studies provide
- This paper presents the potential applications and the hurdles facing anti-HCV siRNA drugs.
- The present review provides insight into the feasible therapeutic strategies of siRNA technology, and its potential for silencing genes associated with HCV disease.
- A basic problem in the design of xx is presented by the choice of a xx rate for the measurement of experimental variables.
- This paper examines a new measure of xx in xx based on fuzzy mathematics which overcomes the difficulties found in other xx measures.
- This paper describes a system for the analysis of the xx.
- The method involves the construction of xx from fuzzy relations.
- The procedure is useful in analyzing how groups reach a decision.
- The technique used is to employ a newly developed and versatile xx algorithms.
- The usefulness of xx is also considered.
- A brief methodology used in xx is discussed.
- The analysis is useful in xx and xx problem.
- A model is developed for a xx analysis using fuzzy matrices.
- Algorithms to combine these estimates and produce a xx are presented and justified.
- The use of the method is discussed and an example is given.
- Results of an experimental applications of this xx analysis procedure are given to illustrate the proposed technique.
- This paper analyses problems in
- This paper outlines the functions carried out by ...
- This paper includes an illustration of the ...
- This paper provides an overview and information useful for approaching
- Emphasis is placed on the construction of a criterion function by which the xx in achieving a hierarchical system of objectives are evaluated.
- The main emphasis is placed on the problem of xx
- Our proposed model is verified through experimental study.
- The experimental results reveal interesting examples of fuzzy phases of : xx,xx
- The compatibility of a project in terms of cost, and xx are likewise represented by linguistic variables.
- A didactic example is included to illustrate the computational procedure

Introduction 引证核心文献，提出假设，指出文章的核心观点

Beginning

- Over the course of the past 30 years, .. has emerged form intuitive
- We evaluated 508 participants who
- Acute kidney injury (AKI) is associated with an increased incidence of respiratory failure requiring mechanical ventilation, which greatly increases mortality
- The cause of respiratory failure in patients with AKI is incompletely understood
- However, lung injury also occurs after ischemia–reperfusion injury of other organs such as the liver, gut, and hind limb
- We have demonstrated previously that
- Given this background, we hypothesized that
- we demonstrate that
- Technological revolutions have recently hit the industrial world
- The advent of ... systems for has had a significant impact on the
- The development of ... is explored
- The concept of xx was investigated quite intensively in recent years
- There has been a turning point in ... methodology in accordance with the advent of ...
- A major concern in ... today is to continue to improve...
- It has become increasingly clear that
- In this paper, we focus on the need for
- This paper proceeds as follow.
- The structure of the paper is as follows.

Our study

- In this paper, we shall first briefly introduce…
- To begin with we will provide a brief background on the
- This will be followed by a description of the xx of the problem and a detailed presentation of how the required membership functions are defined.
- Details on xx and xx are discussed in later sections.
- Polyphenolic compounds are vasodilators and help to lower the risk of cardiovascular diseases.
- Taken together, our novel findings suggest that the EDR induced by the strawberry extract was mediated by activation of the PI3 kinase/Akt signaling pathway, resulting in phosphorylation of eNOS.

Objective / Goal / Purpose

- The purpose of the inference engine can be outlined as follows:
- The ultimate goal of the xx system is to allow the non;experts to utilize the existing knowledge in the area of manual handling of loads, and to provide intelligent, computer;aided instruction for xxx.
- The paper concerns the development of a xx
- The scope of this research lies in
- The main theme of the paper is the application of rule;based decision making.
- These objectives are to be met with such thoroughness and confidence as to permit ...

- The objectives of the ... operations study are as follows:
- The primary purpose/consideration/objective of
- The ultimate goal of this concept is to provide
- The main objective of such a ... system is to
- The aim of this paper is to provide methods to construct such probability distribution.
- In order to achieve these objectives, an xx must meet the following requirements:
- In order to take advantage of their similarity
- more research is still required before final goal of ... can be completed
- In this trial, the objective is to generate...
- for the sake of concentrating on ... research issues
- A major goal of this report is to extend the utilization of a recently developed procedure for the xx.
- For an illustrative purpose, four well;known OR problems are studied in presence of fuzzy data: xx.
- This illustration points out the need to specify
- Recent studies have further defined the role of SBP-2 in promoting UGA read-through,
- This concept has been further validated with the discovery of patients with impaired deiodinase activity due to a mutation in SBP-2
- The ultimate goal is both descriptive and prescriptive.
- A wealth of information is to be found in the statistics literature, for example, regarding xx
- This review will focus on the most recent progress achieved in this field, particularly the cellular and molecular aspects of local control of thyroid hormone signaling provided by deiodinases.
- A considerable amount of research has been done .. during the last decade
- A great number of studies report on the treatment of uncertainties associated with xx.
- There is considerable amount of literature on planning
- However, these studies do not provide much attention to undertainty in xx.
- Since then, the subject has been extensively explored and it is still under investigation as well in methodological aspects as in concrete applications.
- Many research studies have been carried out on this topic.
- Problem of xx draw recently more and more attention of system analysis.
- Attempts to resolve this dilemma have resulted in the development of
- Many complex processes unfortunately, do not yield to this design procedure and have, therefore, not yet been automated.
- Most of the methods developed so far are deterministic and /or probabilistic in nature.
- The central issue in all these studies is to
- The problem of xx has been studied by other investigators, however, these studies have been based upon classical statistical approaches.
- Applied ... techniques to
- Characterized the ... system as
- Developed an algorithm to
- Developed a system called ... which

- Uses an iterative algorithm to deduce
- Emphasized the need to
- Identifies six key issues surrounding high technology
- A comprehensive study of the .. has been undertaken
- Much work has been reported recently in these filed
- Proposed
- Presented
- State that
- Point out that the problem of
- Described
- Illustrated
- Indicated
- Has shown / showed
- Address
- Highlights
- A study on ...was done / developed by []
- Previous work, such as [] and [], deal only with
- The approach taken by [] is
- The system developed by [] consists
- A paper relevant to this research was published by []
- []'s model requires consideration of ..
- []' model draws attention to evolution in human development
- []'s model focuses on...
- Little research has been conducted in applying ... to
- The published information that is relevant to this research...
- This study further shows that
- Their work is based on the principle of
- More history of ... can be found in xx et al. [1979].
- Studies have been completed to established
- The ...studies indicated that
- Though application of xx in the filed of xx has proliferated in recent years, effort in analyzing xx, especially xx, is lacking.

提出 Problem / Issue / Question 或假设

- Unfortunately, real-world engineering problems such as manufacturing planning do not fit well with this narrowly defined model. They tend to span broad activities and require consideration of multiple aspects.
- Remedy / solve / alleviate these problems
- It has recently been reported that
- ... is a difficult problem, yet to be adequately resolved
- Two major problems have yet to be addressed
- An unanswered question
- This problem in essence involves using x to obtain a solution.

- An additional research issue to be tackled is ....
- Some important issues in developing a ... system are discussed
- The three prime issues can be summarized:
- The situation leads to the problem of how to determine the ...
- There have been many attempts to
- It is expected to be serious barrier to
- It offers a simple solution in a limited domain for a complex problem.
- There are several ways to get around this problem.
- As difficult as it seems to be, xx is by no means new.
- The problem is to recognize xx from a design representation.
- A xx problem can trace its roots to xx.
- xx [1987] used a heuristic approach to simplify the complexity of the problem.
- Several problems are associated with them.
- Although some progress has been made in this area, at least two major obstacles must be overcome before a fully automated system can be realized.
- Most problems in practice are complicated
- More problem surface here.
- Hamper effort toward a xx system
- In order to overcome the limitations due to incomplete and imprecise xx knowledge, a xx program has been developed, which bases its knowledge upon the statistical analysis of a sample population of xx
- The above difficulties are real challenges faced by researchers attempting to develop
- This type of mapping raises no controversy to the issue of membership function determination.
- However, attempts to quantify the xx have met both theoretical and empirical problems.
- It has become apparent that in order to apply this new methodological framework to real;world problems and data, we have to pay attention to the problems of xx and xx.

MATERIALS AND METHODS

Materials

- Chemicals were purchased from Sigma (St Louis, MO), if not stated otherwise. Experiments were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.
- CsA, EGF, PD98059, U0126, AG1478, Wortmannin, and LY294002 were from Calbiochem (San Diego, CA, USA). Anti-ERK1/2 and anti-Ras were from Transduction Laboratories (Franklin Lakes, NJ, USA). Anti-phospho Raf-1 (Ser^{259}), anti-phospho Raf-1 (Ser^{338}), anti-phospho PKB/Akt (Ser^{473}), anti-PKB, anti-phospho EGFR (Tyr^{1068}), anti-phospho ERK1/2 (Thr^{202}/Tyr^{204}), anti-PI3K 110α, anti-p53, and anti-phospho MEK1/2 ($Ser^{217/221}$) were from Cell Signalling (Danvers, MA, USA). Anti-MEK and anti-Raf-1 (C12) were from Santa Cruz (Santa cruz, CA, USA). Apigenin and all other reagents were from Sigma (Saint Louis, MO, USA).

Animal

- Animal mode. Male Sprague–Dawley rats, originally weighing 100–130 g and finally weighing 290–310 g, were used in this study. Animals used in this study were maintained in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996). All animals had free access to water and a standard laboratory diet, and were raised at room temperature (23– 26 °C) with a 12-h light-dark cycle.
- For IHA hypoxia studies, rats were exposed to a hypobaric chamber and were maintained at 5000 m altitude (PB = 404 mmHg, PO2 = 84 mmHg) in daylight for one 6-h period each day for 42 days [5,8,9,16]. Age-matched control (normoxic) animals were kept in a normoxic environment for a corresponding period.
- Eight- to ten-week-old male C57BL/6 mice (wild-type) and IL-6-deficient mice backcrossed over eight generations on a C57BL/6 background were used
- Mice were maintained on a standard diet and water was made freely available.
- All experiments were conducted with adherence to the NIH Guide for the Care and Use of Laboratory Animals.
- The animal protocol was approved by the Animal Care and Use Committee of the University of Colorado
- Three surgical procedures were performed as described previously:[5] (1) sham operation, (2) ischemic AKI, and (3) bilateral nephrectomy.
- The abdomen was closed in one layer.
- Sham surgery consisted of the same procedure except that clamps were not applied.
- For bilateral nephrectomy, renal pedicles were tied off with suture and then cut distally.
- The ureters were pinched off with forceps and the kidneys removed.
- Serum was collected as described previously.[5] Blood urea nitrogen and creatinine were measured using an autoanalyzer (Beckman Instruments, Fullerton, CA, USA).
- Serum IL-6 was measured by ELISA according to assay instructions (R&D Systems, Minneapolis, MN, USA).
- Five-micrometer sections of paraffin-embedded lung tissue were stained with hematoxylin and eosin using standard protocols. Neutrophils were counted on the basis of morphological criteria; at least 50 high-powered fields (×40) were counted per slide.
- Frozen lung was prepared for ELISA as described previously.[5] Supernatants were analyzed for protein content using a Bio-Rad DC protein assay kit (Hercules, CA, USA). KC and MIP-2 were determined by ELISA (R&D Systems, Minneapolis, MN, USA).
- One-fourth lung was used to determine MPO activity as described previously.
- Frozen lung was homogenized in radioimmunoprecipitation assay buffer with protease inhibitor; western blotting was performed as described previously.[49] Goat anti-murine ICAM-1 polyclonal antibody (R&D Systems, Minneapolis, MN, USA; 1:2000) or rat anti-murine VCAM-1 monoclonal antibody (R&D Systems; 1:1000) were used.
- A total of 20 μg anti-IL-6 antibody vs IgG control (eBioscience, San Diego, CA, USA) was administered to wild-type mice by tail vein injection 1 h before surgery,

intraperitoneally at the time of clamp removal (ischemic AKI) or nephrectomy (bilateral nephrectomy) and intraperitoneally 1 h following surgery (60 μg total).

Experimental groups

- STZ-induced diabetic rats, a model of partial type I diabetes: SD rats received a single intraperitoneal injection of freshly prepared STZ (65 mg kg-1 body weight, dissolved in 100 mmol l-1 citric acid, pH 4.5), and confirmed 2 days later by PP blood glucose (>250 mg dl-1).
- CTR rats: Vehicle-injected SD rats after 2 to 7 days, 14 to 30 days, and 90 days served as CTR for the 2 and 7 days STZ, the 14 and 30 days STZ, and for the 90 days STZ, respectively.
- Insulin treatment in STZ: Glc was normalized in seven animals during 12–14 days of STZ by subcutaneous insulin implants (2U day-1; Lin Shin Canada, Ontario, Canada).

Cell Culture

- Immortalized cells from the convoluted portion of mouse kidney proximal tubule PKSV-PCT cells (PCT3 clone) were cultured in a medium A (DMEM/Ham's F12 (1:1, v/v), 20 mM HEPES, 2 mM L-glutamine, 12.5 mM D-glucose, 60 nM sodium selenite, 5 $\mu g\ ml^{-1}$ transferrin, 50 nM dexamethasone, 100 $U\ ml^{-1}$ penicillin, and 100 $\mu g\ ml^{-1}$ streptomycin), supplemented with 2% fetal bovine serum, 5 $\mu g\ ml^{-1}$ insulin, 10 $ng\ ml^{-1}$ EGF, and 1 nM triiodothyronine at 37°C in a 95:5 air/CO_2 water-saturated atmosphere. For all experiments, cells were seeded at 0.2 $\times 10^6$ cells/ml and after 24 h with complete medium cells were starved for 16 h in medium A supplemented with 0.1% fetal bovine serum but not insulin, EGF, or triiodothyronine. CsA was dissolved in ethanol and all the pharmacological inhibitors were in DMSO. In all cases, controls were carried out with cells treated with the corresponding vehicle alone. After treatments, cells were washed twice with cold phosphate-buffered saline (PBS) and harvested with lysis buffer as in Llorens *et al*

Isolation of primary bone marrow cells for MSCs. C57BL/6 actin-GFP mice (003291; Jackson Laboratories) were sacrificed; tibiae, femurs, and spine were removed and excess soft tissue was eliminated. Using a pestle and mortar, the bones were crushed and washed in PBS with 0.5% FBS and passed through a 70-μm filter into a collection tube. The slurry was spun at 470 *g* for 5 minutes; the supernatant was removed, and cells were resuspended in a minimal volume of ACK lysing buffer (Cambrex) for 4 minutes on ice and washed once with PBS. After pelleting once again, the cells were resuspended and plated in α20% and incubated at 33°C with 5% CO_2. After 4 weeks of culture and expansion, CD105 isolation was performed by magnetic isolation (Dynabeads M-280 Streptavidin; Invitrogen) or MACS beads (Miltenyi Biotech) using an anti-mouse CD105 biotin antibody (clone MJ7/18; eBioscience) at 10 μg/ml. The CD105-positive cells were then maintained in α20% as before. Passage number for experiments has been indicated below.

Cell viability

- After treatments, PCT3 cells were harvested and washed twice with cold PBS, and the viable cells were counted with Trypan Blue Dye (Gibco-Life Technologies, Grand Island, NY, USA) in a Neubauer chamber. Living cells exclude the dye, whereas dead cells will take up the blue dye. For Hoechst staining, cells seeded in six-well dishes were washed twice with PBS and fixed for 15 min with 4% paraformaldehyde at room temperature. Then, cells were washed twice again with PBS and stained with Hoescht (5 μg ml-1 in PBS) for 5 min.

Alkaline phosphatase staining. Purified $CD105^+$ cells (passages 3–5) were isolated by MACS beads using a biotinylated CD105 antibody and plated at 2×10^3 cells/well in a 96-well plate (BD Biosciences) at 33° in osteogenic induction medium: α20% modified with glycerol 2-phosphate (2.16 mg/ml), 2-phospho-L-ascorbic acid (0.05 mg/ml), and dexamethasone (10 nM) (Sigma-Aldrich). After 18 hours of differentiation, alkaline phosphatase staining was carried out with BCIP/NBT solution (Sigma-Aldrich) per the manufacturer's instructions.

In situ collagen I staining. For in situ collagen I staining, cells treated for 5 days with Bzb or DMSO were washed with PBS/0.5% FBS and exposed for 15 minutes to a rabbit anti-mouse collagen I antibody (21286; Abcam) diluted 1:200 in PBS. After incubation, the wells were washed 3 times and the secondary antibody, Alexa Fluor–conjugated anti-rabbit IgG, was added at 1:200. Wells were again washed 3 times and finally fixed with 2% PFA for 10 minutes prior to visualization by microscopy. Brightly positive collagen I clusters were quantified for each well.

In situ collagen I staining. For in situ collagen I staining, cells treated for 5 days with Bzb or DMSO were washed with PBS/0.5% FBS and exposed for 15 minutes to a rabbit anti-mouse collagen I antibody (21286; Abcam) diluted 1:200 in PBS. After incubation, the wells were washed 3 times and the secondary antibody, Alexa Fluor–conjugated anti-rabbit IgG, was added at 1:200. Wells were again washed 3 times and finally fixed with 2% PFA for 10 minutes prior to visualization by microscopy. Brightly positive collagen I clusters were quantified for each well.

Mice, surgery, and transplantation. For the moderate limb ischemia model, recipient mice were C57BL/6 males (age 12–16 weeks). Ischemia was induced under anesthesia (100 mg/kg ketamine plus 10 mg/kg xylocaine) by ligation of the left and, where indicated, right common femoral arteries. Cells (1×10^6 in total), resuspended in PBS, or PBS alone were injected in equal fractions in the adductor and lower calf region (Supplemental Figure 1, A and B), always on the left side, immediately after ligation.

For the severe limb ischemia model, recipient mice were BALB/c-*nu/nu* males (age 12–16 weeks). Ischemia was induced under anesthesia (100 mg/kg ketamine plus 10 mg/kg xylocaine) by ligation/transection of the left iliac artery (laser Doppler studies) or bilateral ligation/transection of the common femoral arteries (treadmill studies). Cells (1×10^6 in total), resuspended in PBS, or PBS alone were injected as described above (Supplemental Figure 1, A and B);

however, cells were injected 5 days after surgery, and for treadmill studies, both limbs were transplanted.

As MAPC-U do not express *MHC-I* and, consequently, are sensitive to NK cell-mediated clearance, all mice were injected i.p. with 20 µl anti-asialo GM1 antibodies (20× diluted in PBS; Wako Chemicals) 1-2 hours before transplantation and every 10 days thereafter. These antibodies selectively eliminate NK cells without affecting the macrophage or lymphocyte function (47). Mice were housed in specific pathogen-free conditions, and procedures involving animals were approved by the Institutional Animal Care and Use Committees of the Universities of Minnesota, Navarra, and Leuven.

Langendorff heart perfusion

Anesthesia was induced with sodium pentobarbital (45 mg/kg, i.p). The hearts were rapidly excised and perfused with Krebs–Henseleit solution at 37 °C using the Langendorff technique at a constant pressure of 80 mmHg, as previously described in [5,9]. The perfusion solution containing (in mM) 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 0.026 Na_2EDTA, and 11.1 glucose was gassed with 95% O_2–5% CO_2 (pH 7.4). A waterfilled latex balloon connected to a pressure transducer (Gould P23Db) was inserted into the left ventricle (LV). The balloon was slightly larger than the ventricular cavity to achieve a stable LV end-diastolic pressure (LVEDP) of about 8 mmHg during initial equilibration. Heart rate, LV peak systolic pressure (LVPSP), LVEDP and LV developed pressure (LVDP) were monitored on a PowerLab system (AD Instrument Ltd., Australia). The product of heart rate and LVDP (PRP) was calculated (PRP = HR × LVDP). After 15 min of stabilization, the heart was subjected to 30 min of global no-flow ischemia, followed by 120 min of reperfusion. During ischemia, the heart was immersed in the ungassed preischemic coronary perfusate to maintain the temperature at 37 °C. When added, CsA (0.2 µM) or atractyloside (20 µM) was introduced at the beginning of 15 min of reperfusion [17]. At the end of the experiment, the heart was rapidly removed from the Langendorff apparatus and frozen in liquid nitrogen for infarct size analysis.

2.3. Measurement of infarct size

The frozen hearts were cut into 1-mm thick slices. They were then incubated in 1% w/v triphenyltetrazolium chloride (TTC; phosphate buffer, pH 7.4) 15 min for staining. Slices were fixed in 10% formalin to enhance the contrast between stained viable and unstained necrotic tissue. Infarct size was calculated using Adobe Photoshop 5.0, and the infarct area was expressed as a percentage of LV area.

Preparation of adult rat ventricular myocytes

Hearts were retrogradely perfused with oxygenated Ca^{2+}-free Tyrode's solution (37 °C, pH 7.4) for 5 min. The perfusion solution was then switched to an enzyme solution consisting 1% collagenase I, 0.01% protease (type XIV), and 0.08 mM $CaCl_2$. Perfusion was terminated when the tissue became soft (approximately 15 min). The LVwas then minced and filtered through nylon mesh. The cells were resuspended in a series of Tyrode's solutions with gradually increasing Ca^{2+} concentrations (0.5, 1.0, 1.5, and ultimately 1.8 mM). Finally, the myocytes were suspended in approximately 20 ml Tyrode's solution.

Confocal imaging for detecting MPTP opening in the isolated myocyte

The model for detecting MPTP opening in the intact cell was as described by Hausenloy et al. [18]. Cells were incubated with 3 µMtetramethylrhodamine methyl ester (TMRM) for 15 min at 37 °C. After loading, the cells were washed twice with Tyrode's solution. The TMRM-loaded myocytes were placed in a chamber and mounted

on the stage of a Bio-Rad Radiance 2100 confocal microscope equipped with × 40 oil immersion and quartz objective lens (NA 1.3). The cells were illuminated using the 543-nm emission line of a He/Ne laser. All conditions of the confocal imaging system (laser power, confocal pinhole, pixel dwell time, and detector sensitivity) were identical to ensure comparability between experiments. The fluorescence ofTMRMwas collected using a 570-nm long pass filter. Images were analyzed using Bio-Rad software (Lasersharp2000). TMRMgenerates reactive oxygen species (ROS) with continuous laser illumination [19]. Laser-induced oxidative stress was continued until MPTP opening had been provoked, and then continued until rigor contracture took place. As the oxidative stress generated at reperfusion also involves excess production of ROS from mitochondria, this should represent a useful model for reperfusion-induced cell injury [18,20].We measured the time taken to induce the initiation of MPTP opening and the time taken to induce the initiation of rigor contracture.

Isolation of heart mitochondria

Mitochondria were isolated by differential centrifugation from rat hearts. The LVwas homogenized in ice-cold medium containing (in mM) 180 KCl, 20 Tris–HCl, 1 EGTA (pH 7.7), and 0.5% fatty-acid-free BSA. After centrifugation of the homogenate at 1000 × *g* for 10 min, the supernatant was centrifuged at 8000 × *g* for 10 min. The crude mitochondria were resuspended and centrifuged at 8000 × *g* for 10 min. Mitochondria were resuspended in the storage buffer (in mM) 180 KCl, 20 Tris–HCl, and 1 EGTA at pH 7.35. Protein concentrations were determined by the Bradford method, using BSA as a standard.

Measurement of mitochondrial respiratory function

Mitochondria (0.5 mg/ml protein) were suspended in 1 ml incubation buffer (in mM) 110 KCl, 10 mannitol, 10 Tris– HCl, 1 KH_2PO_4, 50 creatine, 5 nitrilotriacetic acid (NTA), and respiration substrate 1 pyruvate + 1 malate at pH 7.2 25 °C. NTA was present to allow buffered [Ca^{2+}] of 50–250 μM to be employed. Modifications of the medium (i.e. extra-mitochondrial) Ca^{2+} and Mg^{2+} concentrations were calculated as stated previously [21]. State 3 (oxygen consumption stimulated by adding 1 mM ADP) and state 4 (oxygen consumption after completion ofADP phosphorylation) were measured using a Clark type electrode (SP-2, Institute of Plant Physiology and Ecology, ChineseAcademy of Sciences) [21]. The respiratory control rate (RCR) was then calculated by comparing the state 3 rate with the state 4 rate. The ADP/O ratio was calculated as the ratio of nanomoles of ADP added to nanoatoms of oxygen consumed during state 3 respiration. *2.9. Ca^{2+}-induced MPTP opening*

Mitochondria (0.5 mg protein) were suspended in 1 ml incubation buffer in a stirred cuvette at 37 °C and the change in absorption at 540 nm was recorded continuously using a spectrophotometer (Lambda2S, USA). After 1 min preincubation, the energized mitochondria were induced to swell with 50, 100, and 200 μM $CaCl_2$. In order to relate MPTP opening to absorption changes, we added 0.2 μM CsA to inhibit MPTP opening or 20 μM atractyloside to abolish any

observed reduction.

Immunohistochemistry.

- Sections of paraffin-embedded jejunum (10 μm thick) were dewaxed in xylene and rehydrated, and antigen retrieval was performed by heating of sections with 10 mM citrate buffer, pH 6, for 15 minutes in a microwave. Sections were washed in PBS and blocked for 30 minutes in 1% BSA, 0.02% Triton X-100, and 10% goat serum. Serial sections were incubated with both primary antibodies against goat polyclonal T2R138 (1:100; Santa Cruz Biotechnology Inc.) and rabbit polyclonal chromogranin A (1:100; Abcam), washed in PBS 3 times for 15 minutes, and incubated with Alexa Fluor

488–conjugated donkey anti-goat secondary antibody (Invitrogen) and Cy3-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories Inc.). The sections were washed in PBS 3 times for 15 minutes and mounted by VECTASHIELD (Vector laboratories). Images from serial sections were acquired by an inverted microscope Axioskop using the AxioVision camera and software (Zeiss).

Western blots/ Immunoblot

- The protein content of cellular extracts was quantified by the Bradford assay.44 Twenty-five microgram of total cell extract protein was run on SDS-polyacrylamide gel electrophoresis gels, transferred onto polyvinylidene difluoride membranes, and incubated with the corresponding antibodies. The membranes were developed with the enhanced chemiluminescence method (Pierce, Rockford, IL, USA).
- Supernatants of growing or growth-arrested cells were centrifugated for 5 min at 10 000 *g*. The cells were lysed as described. The proteins from supernatant and cell lysates were concentrated using heparin sepharose. The heparin sepharose was washed four times with phosphate-buffered saline containing protease inhibitors, dissolved in phosphate-buffered saline/protease inhibitor and incubated with 500 µg protein over night at 4°C. The complexes were washed with phosphate-buffered saline/protease inhibitor and the proteins were eluated with 100 µl Laemmli buffer without bromophenol blue (10 min 95°C). A 30 µl probe was loaded in each lane and western blot analysis was performed as described, using a polyclonal antibody against CCN3 (K19M), which recognizes a C-terminal 19-aminoacid peptide of human CCN3. As a positive control, a supernatant from adrenocortical cell cultures, which are known to secrete CCN3, was used.
- Cells were lysed in 0.5% (volume/volume) Triton X-100 lysis buffer and immunoblot analysis was done as described[43]. Immunoprecipitation with anti-CrkL or control rabbit antiserum was done as described[44]. Antibodies to the following were used: phosphorylated Erk (910L; Cell Signaling); phosphorylated Jnk (V7932; Promega); Erk (13-6200; Zymed); Jnk1 (sc-474), H-Ras (sc-35), C3G (sc-869), CrkL (sc-319), RasGRP1 (sc-8430) and DGK-ζ (sc-8722; all from Santa Cruz Biotechnologies); and DGK-α (a gift from H. Kanoh, Sapporo Medical University, Sapporo, Japan). Images were scanned, followed by densitometry analysis with UN-SCAN-IT software (Silk Scientific).
- Purified splenic T cells were stimulated for various times with 5 µg/ml of anti-CD3ε (500A2; BD Pharmingen) and were lysed in 1% Nonidet P-40 lysis buffer (1% (volume/volume) Nonidet-40, 150 mM NaCl and 50 mM Tris, pH 7.4) with protease inhibitors. Proteins were resolved by SDS-PAGE and were transferred to a Trans-Blot Nitrocellulose membrane (Bio-Rad Laboratories); membranes were probed with antibodies specific to phosphorylated Erk (91015; Cell Signal Technology) and phospholipase C-γ1 (05-163; Upstate Biotechnology). Membranes were stripped and were reprobed for analysis of total Erk (SC-16982; Santa Cruz Biotechnology). Activated Ras in cell lysates was determined by glutathione *S*-transferase–Raf—Ras-binding domain precipitation assay as described.

- ***SDS-PAGE and Western blotting.*** A single-cell suspension was prepared from spleens incubated for 1 hour with collagenase as described previously (67). Splenocytes were washed with PBS and lysed with RIPA buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% NaDeoxycholate, 1% Triton X-100) plus protease (Roche) and phosphatase inhibitors. Protein concentrations were determined by the DC protein assay (Bio-Rad). Fifty micrograms of protein per sample were resolved by SDS-PAGE and analyzed by Western blotting using anti-NFATc1 (mAb 7A6; BD Biosciences — Pharmingen) and rabbit anti-mouse HSP90 (Santa Cruz Biotechnology Inc.) antibodies along with the appropriate HRP-conjugated secondary reagents.
- ***Immunoblotting analysis.*** Colon tissues were homogenized and sonicated in RIPA lysis buffer (Santa Cruz Biotechnology Inc.), supplemented with protease inhibitors. After centrifugation at 20,000 *g* for 15 minutes, 30 μg of the supernatants were separated on 10% SDS-polyacrylamide gel and transferred onto an Immobilon-P Transfer membrane (Millipore). After being blocked with 5% skim milk, the membrane was incubated with antibodies to total β-catenin (1:1,000) and active β-catenin (1:500). Rabbit anti–α/β-tubulin antibody (1:1,000) was used as an internal control. ImmunoPure peroxidase-conjugated anti-mouse or anti-rabbit IgG were used as secondary antibodies. The blotted membrane was then treated with the Super Signal West Dura Extended Duration Substrate (Pierce Biotechnology Inc.) and signals were detected by LAS-3000 mini CCD camera (Fuji Film).

Immunofluorescence microscopy.

- Analysis of protein localization in 2C T cell–P815.B71 cell conjugates was done as described[29]. P815.B71 cells were labeled with CMAC (7-amino-4-chloromethylcoumarin) Cell-Tracker Blue (Molecular Probes) and were mixed with equal numbers of anergic or *in vitro*–primed 2C *Rag2*$^{-/-}$ T cells. After approximately 8 min, cells were fixed, were made permeable and were stained with anti-GRP1 and anti-talin (Santa Cruz Biotechnologies) and with species-specific secondary antibodies conjugated to fluorescein isothiocyanate or phycoerythrin, respectively. Samples were analyzed with a Zeiss Axiovert 100 microscope, and 15 conjugates were typically assigned scores. Slidebook software (Intelligent Imaging Innovations) was used for image capture and deconvolution analysis. ImageJ 1.36b software (US National Institutes of Health) was used for quantification of pixel intensity.
- ***Immunofluorescence of NFATc1.*** BMOcPs were cultured on chamber slides for 3 days with M-CSF and RANKL. Cells were fixed with 4% PFA, permeabilized, and stained with anti-NFATc1 and goat anti-mouse Alexa Fluor 488 (Invitrogen). Hoechst (Sigma-Aldrich) was used as a nuclear counterstain.

Measurement of ROS generation

- The assay is based on the incorporation of 2',7'-dichlorofluorescein diacetate into the cell. H2O2 and peroxidases are able to oxidize the cleaved DCFH to DCF, which is highly fluorescent at 530 nm. To measure CsA-induced ROS generation, cells were washed twice with PBS, and fresh medium containing 20 μM 2',7'-dichlorofluorescein diacetate was added to previously treated cells. After 30 min cells were washed again, tripsinized,

and resuspended with cold PBS. Fluorescence was measure by flow cytometry on a FACScan flow cytometer.

Raf-1 activity

- Raf-1 immunoprecipitation and kinase assay were performed as described previously.45 Immunoprecipitated Raf was incubated for 30 min at 30°C with 0.8 mM ATP, 10 μg ml-1 GST-MEK, and 100 μg ml-1 GST-ERK2. An aliquot of the supernatant was used for ERK2 activity assays using 0.5 mg ml-1 myelin basic protein and 0.1 mM [γ-32P] ATP (400 c.p.m. pmol-1). After 15 min incubation at 30°C, 12 μl of 5 ×Laemmli loading buffer was added to the tubes and the mixture analyzed by SDS-polyacrylamide gel electrophoresis. Radiolabeled bands were quantified in a PhosphoImager.

Semiquantitative RT-PCR.

- Total RNA was isolated from freshly isolated thymocytes. Then, cDNA was prepared with the M-MuLV reverse transcriptase and random primers according to the manufacturer's recommendations (New England Biolabs). Semiquantitative PCR analysis of *Tcrb* VDJC (where 'C' is the constant region) and *Cd3e* cDNA was done as described[51]. [^{32}P]dCTP (GE Healthcare Life Science) was incorporated into PCR products for semiquantitative detection by autoradiography.

Real-time quantitative RT-PCR

- Total RNA was isolated from HMC or rat mesangial cells using the Invisorb Spin Cell-RNA Mini Kit (Invitek, Berlin, Germany) or from isolated glomeruli using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA purity determination, cDNA synthesis, and RT-PCR were performed as described.16 Primer sequences are listed in Table 2. Glyceraldehyde-3-phosphate dehydrogenase cDNA amplification was used as an internal standard.
- Total RNA was isolated from the frozen kidneys as described by Chomczynski and Sacchi47 and quantified by a photometer. One microgram of the resulting RNA was used for reverse transcriptase (RT)-PCR. The cDNA was synthesized by MMLV reverse transcriptase (Superscript-Invitrogen, Carlsbad, CA, USA). For quantification of renin mRNA expression (sense: 5'-ATGAAGGGGGTGTCTGTGGGGTC-3', antisense: 5'-ATGCGGGGAGGGTGGGCACCTG-3'), real-time RT-PCR was performed using a Light Cycler Instrument (Roche Diagnostics Corp., Basel, Suisse) and the QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany), with GAPDH (sense: 5'-TTCATTGACCTCAACTACAT-3', antisense: 5'-GAGGGGCCATCCACAGTCTT-3') as a control. PCR was run for 30 cycles with 15 s per 95°C denaturation, 20 s/58°C annealing and 20 s/72°C elongation. To verify the accuracy of the amplicon, a melting curve analysis was done after amplification.Total renin mRNA content per kidney was calculated from the yield of RNA extracted from the whole kidneys times the renin mRNA estimate obtained from the defined amount of RNA used for RT-PCR real time measurement. For the RT-PCR real-time measurements, a pool of RNA from adult mouse

kidneys was generated, which served as standard for all RT-PCR runs. Thus, all renin mRNA levels for the developing kidneys were estimated relative to the levels in adult kidneys.

- Total RNA was extracted using either RNeasy kits (Qiagen) or TRIzol (Invitrogen) and solubilized in RNAase-free water. cDNA was synthesized using either kits from Stratagene or Bio-Rad. qRT-PCR reactions were performed using the SYBR Green PCR Master Mix (Applied Biosystems) or the Brilliant II SYBR Green QPCR Master Mix (Stratagene) in a PRISM 7700 system (Applied Biosystems) or Mx3005P QPCR System (Stratagene). Ct values for duplicate samples were averaged and the amounts of mRNA relative to Hprt were calculated using the ΔCt method. qRT-PCR primers used in this study are reported in Table 1. All qRT-PCR reactions yielded products with single peak dissociation curves.
- *Quantitative RT-PCR.* Total RNA was extracted from colon tissues with RNA-Bee (Tel-Test Inc.) and 2.5 μg of RNA was reverse-transcribed using ReverTraAce (Toyobo) and random primers as described previously. Real-time PCR was performed on Applied Biosystems StepOne Real-Time PCR System (Applied Biosystems) using the comparative C_t quantitation method. TaqMan Gene Expression Assays (Applied Biosystems) containing specific primers (accession numbers: TNF-α - Mm00443258_m1, KC/CXCL1 - Mm00433859_m1, MCP-1/CCL2 - Mm00441242_m1, COX-2 - Mm00478374_m1, GAPDH - Mm99999915_g1), TaqMan MGB probe (FAM dye-labeled), and TaqMan Fast Universal PCR Master Mix were used with 10 ng of cDNA to detect and quantify the expression levels of TNF-α, KC/CXCL1, MCP-1/CCL2, and COX-2 in mouse colon tissues. GAPDH was amplified as internal control. C_t values of GAPDH were subtracted from C_t values of the target genes (ΔC_t). ΔC_t values of treated mice were compared with ΔC_t values of untreated animals. Reactions were done at 95° C for 20 seconds followed by 40 cycles of 95° C for 1 second and 60° C for 20 seconds, 60° C, 20 seconds — 40 cycles.

In vitro anergy assay.

- Wild-type, Dgka-/- and Dgkz-/- splenocytes were stained with 5 μM CFSE, were stimulated for 72 h with anti-CD3 (1 μg/ml; 2C11) along with CTLA-4–Fc (5 μg/ml), were stained with allophycocyanin-conjugated anti-CD4 and were analyzed by flow cytometry. Cell division was assessed by CFSE dilution after gating on live CD4+ cells. Alternatively, cells were stimulated for 72 h and were pulsed with 1 μCi/well of [3H]thymidine for the final 8 h of stimulation, and proliferation was assessed by tritium incorporation with a scintillation counter. For restimulation analyses, cells were prestimulated with anti-CD3 plus CTLA-4–Fc, then after 72 h, CD4+ cells were purified by negative selection (with fluorescein isothiocyanate–conjugated anti-CD8, anti-B220 (RA3-6B2; BD Pharmingen), anti-DX5 and anti-CD11b (M1/70; BD Pharmingen), followed by depletion with anti–fluorescein isothiocyanate magnetic beads) and were allowed to 'rest' overnight at 37 °C. Live cells were then counted by Trypan blue exclusion, and equivalent numbers of live cells were dropped onto monolayers of bone

marrow–derived macrophages coated with anti-CD3 (1 μg/ml) and anti-CD28 (0.5 μg/ml). After 24 h, supernatants were collected and IL-2 was quantified by ELISA according to the manufacturer's protocol (R&D Systems).

Three-dimensional reconstruction

- Serial sections of kidney specimens were fixed and stained for renin and for αSMA as described above. Digitalization of the serial slices was performed using an AxioCam MRm camera (Zeiss, Jena, Germany) mounted on an Axiovert200M microscope (Zeiss) with fluorescence filters for renin and αSMA (TRITC: filter set 43: Cy2: filter set 38 HE; Zeiss). After acquisition, a stack of equal-sized images was built using the graphic tool ImageJ (Wayne Rasband, NIH, Bethesda, MD, USA). The equalized data were then imported into the Amira 4.1 visualization software (Mercury Computer Systems Inc., Chelmsford, MA, USA) on a Dell Precision 690 computer system (Dell, Frankfurt, Germany), and subsequently split into the renin and αSMA channels. After this step, the renin and αSMA channels were aligned. In the segmentation step, the αSMA and renin data sets served as a scaffold and were spanned manually or automatically using grayscale values. Matrixes, volume surfaces, and statistics were generated from these segments.

Histology and 3D reconstruction. Mouse heads were fixed using 4% PFA in PBS and paraffin embedded. Longitudinal sections of 5 μm were cut and stained with PAS to examine the middle ear. Sections used for 3D reconstruction were prepared as above except that fixed tissue was embedded in Araldite resins (48). Serial longitudinal sections of 40-μm thickness were stained with epoxy stain (Electron Microscopy Sciences). One digital image of the area including the middle ear cavity to the nasopharynx was taken for each ear of each section. AMIRA software (Mercury Computer Systems) was used to align the stack for each ear, to select and contour each area of interest (middle ear cavity, eustachian tube, and nasopharynx), and to generate a 3D surface view for each ear. Measurements were made from 3D reconstructed images.

In silico analysis of the Tnfrsf11b promoter and NFATc1 ChIP assay.

- The mouse and human genomic sequences 3.6 kb upstream of the Tnfrsf11b gene were extracted from Ensembl database (www.ensembl.org). Z picture and rVista options in ECR browser (75) were used to identify and align regions of high conservation and putative NFATc1 binding sites in this region, respectively. ChIP assays were performed as described previously (16). Briefly, WT M-CSF–primed spleen cells were incubated with RANKL and immunoprecipitation was carried out on precleared, sheared soluble chromatin with anti-NFATc1 (Santa Cruz Biotechnology Inc.) or nonspecific IgG overnight at 4°C. Immune complexes were isolated using Protein G agarose, washed, decrosslinked, and purified as described previously (16). ChIP samples were analyzed by qRT-PCR for the Tnfrsf11b promoter using the FastStart TaqMan Master kit (Roche) and

the universal probe library (Roche Diagnostics) with the following primers: 5′-TTAGGGAATACCTCAGGAAAATACA-3′ (forward) and 5′-TTGTAGGAGCACGAGGTGAA-3′ (reverse). Analysis for the Itgb3 promoter was performed as described (18). The signal for the promoter being studied was adjusted using input threshold values as a reference and is reported as relative enrichment.

ChIP assays. We proceeded as previously described (58) with minor modifications. Briefly, hepatocyte lineage PLC/PRF/5 cells treated with UDCA and/or dexamethasone were fixed with 1% formaldehyde (10 minutes at room temperature), washed twice with ice-cold PBS, and lysed in 5 mM Tris-HCl pH 8.0, 85 mM KCl, 0.5% NP-40, and CPI. Nuclei were pelleted at 3,000 *g* and resuspended in 50 mM Tris-HCl pH 8.1, 10 mM EDTA, 1% SDS, and CPI. Chromatin was sheared into approximately 500-bp fragments using a Bandelin sonicator (6 cycles at 15 seconds each, at 30% maximum power) followed by centrifugation to pellet debris. Aliquots with supernatant proteins (150 μg in 500 μl of 20 mM HEPES, pH 7.9, 0.5 mM DTT, 20% glycerol, and CPI) were incubated for 15 min at 4° C with 50 μl of protein A/G–agarose beads, BSA, and total IgG from mouse, goat, or rabbit — depending on the host species for immunoprecipitating antibodies (each total IgG was from Santa Cruz Biotechnology Inc.). Immunoprecipitations were carried out overnight at 4° C with the following antibodies (2 μg): rabbit anti-human GR, goat anti-human HNF1α, goat anti-human HNF1β (sc-8992 X, sc-6547 X, sc-7411 X, respectively; all from Santa Cruz Biotechnology Inc.) or mouse anti-human p300 (BD Biosciences — Pharmingen), as well as species-specific total IgG for the negative controls. Immunoprecipitates were collected and washed twice with 2 mM EDTA and 50 mM Tris-HCl pH 8.0 and then 4 times with 100 mM Tris-HCl pH 8.0, 500 mM LiCl, 1% NP-40, and 1% deoxycholic acid. Protein-DNA complexes were then eluted from protein A/G–agarose beads by 2 washings with a freshly prepared solution (50 mM $NaHCO_3$, 1% SDS), with brief shaking and further centrifugation (1,000 *g*, 5 minutes). Collected supernatants were incubated overnight with RNase A (10 μg/ml) at 67° C. Prior to PCR, immunoprecipitates and corresponding input chromatin were digested with proteinase K and extracted with phenol-chloroform as described previously (58). Amplicons $-391b_1/-239b_1$ (which includes GREcore site at $-327b_1$) and $-827b_1/-534b_1$ (with the HNF1 element) were obtained by using the following primers: forward 5′-ACAGGAGCCTTCCTCACAGA-3′ and reverse 5′-CAGAACAAGGAAGAGCGCGGGGAGGGCT-3′ for the former and forward 5′-TAGAGGCGCAGGTCAAGTCT-3′ and reverse 5′-AAGTCCATGGCCGGCAGGGGTGAAC-3′ for the latter. Finally, to amplify a promoter region between HNF1 element and GREcore site at $-327b_1$ (stretch from $-571b_1$ and $-359b_1$), we used 5′-CCCTCCTTCTCAGGTTCACCCCTGCC-3′ and 5′-CTGGCTTCTCCCCTCTGTGA-3′ as forward and reverse primers, respectively. PCR products electrophoresed in agarose gels with ethidium bromide were assessed in a GelDoc XR Analyzer (Bio-Rad).

Preparation of mice for in vivo imaging. To visualize cellular dynamics in vivo, mice were anesthetized by injection with urethane (1.5 g/kg). Our preliminary studies showed that urethane was most suitable to use because in contrast to pentobarbital, isoflurane, barbiturate, and ketamine-xylazine, it did not significantly affect the local microcirculation in the adipose tissue. After the appropriate depth of anesthesia was obtained, a small incision was made and moistened with saline. This window was then covered with Saran Wrap. Intravital imaging was performed through the small (~3 mm) window without exteriorizing the fat pad as shown in Figure 1. Heating pads were used to keep the body temperature at 37° C.

ChIP-chip promoter array.

ChIP assays from mouse liver were performed as previously described (20). To prepare samples for the ChIP-chip array, after reversing the crosslinking and isolating the ChIP-enriched DNA, gene-specific enrichment for HMG-CoA reductase and LDL receptor promoters in the L/E chromatin relative to chow control chromatin was confirmed, and the SREBP-2, IgG control, and input DNA were prepared for hybridization to the 1.5-kb mouse promoter array (NimbleGen/Roche) using a random PCR amplification protocol (41). The hybridization was analyzed by the Signal Map software program from NimbleGen/Roche.

The qPCR primers for the mouse promoters were as follows: HMG-CoA reductase, sense, 5′-GCTCGGAGACCAATAGGA-3′, antisense, 5′-CCGCCAATAAGGAAGGAT-3′; LDL receptor, sense 5′-GAACTTCCCACTGCTGC-3′, antisense, 5′-CACGCCCAGAGTCATTC-3′; T2R138, sense, 5′-TGTCAGCAAGTGCTTCTTGG-3′, antisense, 5′-GGGCTACCCACTCATTCAAA-3′.

-

DNA microarray analysis of MROcPs.

- Independently prepared duplicate samples containing pooled cells from multiple mice for each genotype were analyzed. Briefly, CD11blo/–CD3ε–B220–c-kit+c-fms+ BMOcPs from 8- to 13-week-old Nfatc1Δ/Δ and Nfatc1fl/fl littermate controls (n = 4/genotype, replicate 1 and n = 3/genotype, replicate 2; respectively) were incubated at an initial density of 20,000 per well in 24-well plates, with 1.2 ml αMEM-10 supplemented with M-CSF and RANKL. After 3 days of culture, mRNA was purified (RNeasy; Qiagen) and RNA profiling was performed by the microarray facility at the Harvard Medical School and Partners Center for Genetics and Genomics using the Affymetrix mouse 430A 2.0 array, which contains probes for approximately 14,000 well-characterized mouse genes. Data for all 4 samples were scaled to 500 and replicate samples were averaged for reporting in Figure 4. A gene was classified as “NFATc1 augmented” if (a) the

expression level of that gene was higher in Nfatc1fl/fl samples compared with Nfatc1Δ/Δ samples and (b) a "present" detection call was designated in both samples for the probe set recognizing that gene. "NFATc1 dependent" genes had an "absent" detection call for either one or both of the Nfatc1Δ/Δ samples and were "present" at higher expression levels in Nfatc1fl/fl samples. A similar experimental design was used to generate independent RNA samples for validation of the microarray results by qRT-PCR.

Reverse-phase protein microarray. Cell lysates from cells with and without DOX were adjusted for protein concentrations, denatured with SDS, and serial diluted to define the linear range of each antigen-antibody reaction. Lysates were spotted onto nitrocellulose-coated slides (Whatman) with a G3 microarrayer (Genomic Solutions) and probed with antibodies. Signals were captured by tyramide dye deposition (CSA System; DAKO). Data collected were quantified using quantitating software MicroVigene specifically developed for this approach. Protein phosphorylation levels were expressed as a ratio to equivalent total proteins. The values derived from the slope and the intercepts were expressed relative to standard control cell lysates on the array. All values were compared with the mean within each antibody probing and visualized by heatmaps created from the software MatLab (Mathworks Inc.).

Restimulation assay after *in vivo* immunization.

- For analysis of T cell priming *in vivo*, $CD4^+$ T cells were collected from naive, primed or tolerized recipient mice on day 15 after immunization. Proliferative responses were measured by culture for 72 h of $CD4^+$ T cells (3×10^6 cells/ml) with irradiated (3,000 rads) APCs (10×10^6 cells/ml) and OVA(323–339). The number of KJ1-26^+ cells for each group of recipient mice was determined by flow cytometry and proliferation was normalized to the number of input KJ1-26^+ cells. Supernatants were collected from plates and cytokine concentrations were measured by ELISA.

Flow cytometry.

- For analysis of surface antigen expression, mAb to CD4 (JK1.5; eBioscience) and mAb KJ1-26 (KJ-126; Caltag) were used. For intracellular IL-2 staining, T cells were restimulated for 24 h *in vitro* with OVA(323–339) in the presence of APCs as described above. Brefeldin A (eBioscience) was added for the last 6 h of the culture. Cells were collected and were stained with allophycocyanin-conjugated mAb to CD4 and fluorescein isothiocyanate–conjugated mAb KJ1-26. Then, cells were fixed, were made permeable and were stained with antibody to IL-2 (clone JES6-5H4; eBioscience) according to the manufacturer's instructions.
- T_H1 cells transduced with adenovirus vector encoding GFP were analyzed with a FACScan (BD Biosciences). A total of 1×10^4 events were acquired, and data were analyzed with CellQuest software (BD Biosciences).

- Splenic and lymph node samples depleted of thymocytes and red blood cells were stained with fluorescence-conjugated anti-CD3 (2C11), anti-CD4 (GK15), anti-CD8 (53-6.7), anti-CD25 (7D4) and anti-CD44 (552407; all from BD Pharmingen). A three-color FACScan (Becton Dickinson) was used for flow cytometry, and data were analyzed with FlowJo 4.6 (TreeStar).
- A FACSCalibur (Becton Dickinson) was used for flow cytometry. Human cells from transplanted NOD-SCID mice were assessed with phycoerythrin–cyanin 5–conjugated anti–human CD45 and phycoerythrin-conjugated anti-CD19, anti-CD33, anti-CD36 and anti–glycophorin A (Becton Dickinson). EGFP fluorescence was detected with channel FL1 calibrated to the fluorescein isothiocyanate emission profile. During quadrant analysis, only fluorescence excluding more than 99% of isotypic control events was considered specific. Cell Quest Pro software (Becton Dickinson) and FlowJo (Tree Star) were used for data acquisition and analysis.

Mammalian expression plasmids and transfection.

- For generation of the plasmid expressing Smad3 shRNA, the following specific oligonucleotides were used: upper, 5'-GATCCACCTGAGTGAAGATGGAGATTCAAGAGATCTCCATCTTCACTCAGGTTTTTTTACGCGTG-3'; lower, 3'-AATTCACGCGTAAAAAAACCTGAGTGAAGATGGAGATCTCTTGAATCTCCATCTTCACTCAGGTG-5'. These were cloned under control of the U6 promoter into the pSIREN-DNR-DsRed expression vector (Clontech, BD). Vector expressing shRNA specific for luciferase served as a control. Smad3-Tm was subcloned into the pIRES2-EGFP vector (Clontech, BD); empty vector served as a control. Purified DO11.10 or DO11.10p27Δ T cells were transfected with plasmids by nucleofection with the Amaxa nucleofection apparatus, according to the manufacturer's instructions (Mouse T Cell Nucleofector Kit Amaxa Biosytems). Purified T cells were suspended in nucleofector solution (3×10^6 cells/100 μl) and were mixed with 3 μg of plasmid. Samples were transferred into cuvettes, were transfected with nucleofector program X-01 and were then immediately transferred into 12-well plates and were cultured in nucleofector medium for 3 h. Then, cells were collected and counted and were immediately transferred into syngeneic recipient mice (3×10^6 cells per mouse). At 3 h after adoptive transfer, mice were given priming or tolerizing treatment *in vivo* according to the standard protocol described above. Lymphocytes were isolated from draining lymph nodes at day 5 of the treatment, $CD4^+$ T cells were purified and transfection efficiency was assessed by flow cytometry. The range of transfection efficiency was 69–75% (Supplementary Fig. 4 online). Smad3-knockdown and control-knockdown DO11.10 cells and DO11.10 cells transfected with Smad3-Tm and vector control were selected by cell sorting. The resulting $CD4^+$ T cells (2×10^6 cells/ml) were restimulated with OVA(323–339) (5 μg/ml) in the presence of irradiated APCs *in vitro*.

Plasmids.

- The mouse mT2R138 promoter constructs (–1,780 to +119 and 5′ deletions) were cloned by PCR amplification using mouse genomic DNA as template, followed by recombination with the pDONR201 vector according to Gateway technology (Invitrogen). The mT2R138 construct was transferred by Gateway technology (Invitrogen) into the luciferase reporter vector p-LUC-GW kindly provided by J. Imbert (Institut Paoli-Calmettes, Marseille, France). All constructs were verified by DNA sequencing. The plasmids, 2×flag pCDNA3.1+ SREBP-2 and pSynSRE-positive control SREBP reporter, have been previously described (42).

siRNA transfection and adenovirus infection in STC-1 cells.

- STC-1 cells (ATCC) were maintained in DMEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin plus 50 mmol/l streptomycin) in an atmosphere of 5% CO2 at 37°C. The siRNA targeting mT2R138 (GenBank accession number NM_001001451) and nontargeting siRNA control were purchased from Dharmacon (siGENOME ON-TARGETplus SMART pool L-067135-01-0005 for T2R138). The cells (2 × 105 cells/well) seeded in 12-well plates were transfected for 48 hours with 10 nM of each siRNA using RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions, and dishes were washed and refed with media with or without sterols (12 μg/ml cholesterol and 1 μg/ml 25-hydroxycholesterol) and with or without atorvastatin, as described previously (43). Cells were harvested 24 hours later. For adenovirus infection, STC-1 cells (1 × 106 cells/well) seeded in 6-well plates were infected with Ad-hSREBP-2 (I. Shechter, Uniformed Services University, Bethesda, Maryland, USA), Ad-DN-SREBP (J.B. Kim, Seoul National University, Seoul, Republic of Korea), or Ad-GFP (10 MOI) for 24 hours and collected for analysis after a further 24-hour incubation.

siRNA transfection. SKOv3-ARHI and glioma cells were transfected with control or ARHI siRNAs using the Transfectin 4 reagent (Dharmacon Research). Briefly, a mixture of siRNA (100 nM final concentration) and transfection reagents were incubated for 20 minutes at room temperature. This mixture was then added to cells and allowed to incubate for 48 hours before cells were harvested for protein and RNA expression measurements. For experiments with serial transfections, cells were transfected with siRNA using the Transfectin 4 reagent on day one and with additional siRNA and GFP or GFP-LC3 plasmid using Lipofectamine 2000 (Invitrogen) on day 2. Cells were either harvested 24 hours later for protein expression measurements or fixed for fluorescence microscopy analyses.

Electron microscopy. Tissues were fixed in 2% glutaraldehyde with 0.1 M sodium cacodylate (pH 7.4) for 72 hours at 4°C. Samples were then incubated with 2% osmium tetroxide and 0.1 M sodium cacoylate (pH 7.4) for 1 hour at 4°C. Ultrathin sections were stained with lead citrate and uranyl acetate and were viewed on a JEM 1010 microscope (JOEL).

Scanning electronic microscopy. Skulls from 3-week-old and 16-month-old mice were fixed in 1.5% PFA/2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3). To ensure complete fixation, the external auditory canal was flushed with fixative. After decalcification (0.12M EDTA, pH 7), middle ear cavities were dissected and then gradually dehydrated. Samples were subject to the critical drying point process and

gold coated prior to observation using scanning electron microscopy (Carl Zeiss SMT). Four ears of each genotype were evaluated.

Resin-casted scanning electron microscopy. Femurs were dissected, fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer solution (pH 7.4) at room temperature for 4 hours, and then transferred to 0.1 M cacodylate buffer solution. The specimens were dehydrated in ascending concentrations of ethanol, embedded in methyl-methacrylate, and then surface polished using 1 μm and 0.3 μm alumina alpha micropolish II solution (Buehler) in a soft cloth rotating wheel. The surface was acid etched with 37% phosphoric acid for 10 seconds, followed by 5% sodium hypochlorite for 5 minutes. The samples were then coated with gold and palladium as described previously (48) and examined using an FEI/Philips XL30 Field emission environmental scanning electron microscope.

Luciferase assays.

- CAR IL-2–Luc T_H1 clones were transduced with vectors, were stimulated for 20 h and were resuspended in serum-free DMEM in luminometer cuvettes (BD Biosciences). An equal volume of Bright-Glo luciferase assay reagent (Promega) was added to each sample, followed by thorough mixing. After 2 min, samples were analyzed with a monolight 2010 Luminometer (BD Biosciences).
- ***Luciferase reporter assay for osteocalcin.*** C3H10T1/2 cells (ATCC) were grown in DMEM with 10% FCS. Cells were plated into 12-well plates at 60,000 cells/well. One day after plating, cells were transfected with the indicated combinations of 6xOSE2-*luciferase*, pTK-RL, and *Runx-2* expression constructs using Effectene transfection reagent (QIAGEN). 18 hours later, the medium was changed and the indicated concentration of Bzb was added. 24 hours later, cells were harvested and firefly/renilla luciferase activity was determined per the manufacturer's instructions (Promega). Each experiment was performed in triplicate and repeated at least 3 times. 6xOSE2-luciferase and Runx-2 expression constructs and additional methods are described in ref. 27.

Expression and purification of bacterial GST-tagged proteins The GST-tagged expression constructs were transformed into *E. coli* using ampicillin selection. Bacterial colonies harboring the WT and mutant constructs were grown in LB medium containing 100 μg/ml ampicillin in 1-l flasks at 37°C to a cell density of A_{600} = 0.6. Isopropyl-D-thiogalactopyranoside was then added to 1 mM to induce the expression of GST-tagged proteins, and cultures were grown for 6 hours at 37°C. The bacteria were collected by a 4,000 *g* spin for 15 minutes at 4°C and resuspended in PBS on ice. The bacteria were then lysed on ice by 5 30-seconds sonicator pulses using a virsonic cell disruptor (VirTis Gardiner). The bacterial lysate was then centrifuged at 30,000 *g* for 30 minutes to pellet debris. The fusion proteins were purified from cellular extracts using glutathione-sepharose beads (Amersham Biosciences) according to the manufacturer's instructions, and the GST fusion proteins were eluted with 10 mM glutathione, 50 mM Tris-HCl (pH 7.5), and 120 mM NaCl. Protein purity was assessed by Coomassie-stained SDS-PAGE, and protein concentrations were normalized using the Bradford method. It should be noted that GST-Rac1 fusion proteins containing 88 or 116 amino acids of the N terminus of Rac1 consistently migrated faster than their predicted molecular weights in SDS-PAGE, which was likely due to altered folding properties of domains contained within these deletion mutants. The GST-tagged SOD1 proteins were cleaved from GST using a thrombin cleavage capture kit (EMD Bioscences). Following cleavage, SOD1 proteins were separated from the cleaved GST-tag using an FPLC glutathione-sepharose column.

Nondenaturing gradient gel electrophoresis. Samples of ACM or CSF were mixed 1:1 with native sample buffer and electrophoresed on 4%–20% Tris-Glycine gels (Invitrogen). Proteins with known hydrated diameters were used as size standards (Amersham). Proteins were transferred to a nitrocellulose membrane, probed with a goat anti-mouse apoE antibody at 1:100 (M-20 from Santa Cruz Biotechnology Inc.), washed, and probed with horse anti-goat IgG linked to HRP at 1:1,000 (Vector Laboratories). Bands were visualized with enhanced chemoluminescence (Pierce) and imaged with a Kodak Image Station.

Measurement of apoE protein in brain and CSF. Brain tissue was subjected to a serial extraction method that has previously been used to extract different pools of Aβ (36). Tissue was homogenized in 10 μl/mg carbonate buffer (100 mM sodium, 50 mM NaCl, protease inhibitors, pH 11.5) and centrifuged at 20,000 *g* for 25 minutes. The carbonate extract was collected, and the pellet was rehomogenized in 500 μl of guanidine buffer (5 M guanidine, 50 mM Tris, protease inhibitors, pH 8.0). The guanidine homogenate was rotated for 3 hours at room temperature, then centrifuged at 20,000 *g* for 25 minutes and the guanidine extract collected. apoE in carbonate and guanidine brain extracts, as well as CSF, was measured using a previously described ELISA (18).

Patch clamp recordings

Whole-cell voltage clamp recordings were performed with small-sized DRG neurons and HEK293t cells. Membrane currents were acquired with an Axopatch 200B amplifier (Molecular Devices), filtered at 1 kHz and sampled at 5 kHz. Electrodes were pulled from borosilicate glass tubes (TW150F-3; World Precision Instruments) and heat polished to give a resistance of 1.5–2.0 MΩ. The standard external solution contained 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose (pH 7.4 adjusted with tetramethylammonium hydroxide). In Ca^{2+}-free solutions, $CaCl_2$ was replaced by 5 mM EGTA. The internal solution contained 140 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, and 10 mM HEPES (pH 7.4 adjusted with potassium hydroxide). Unless otherwise noted, cells were held at −60 mV. All recordings were performed at room temperature. Solutions were applied with a polytetrafluoroethylene glass multiple-barrel perfusion system. The heat stimulus was delivered using a multichannel, gravity-driven system incorporating rapid-feedback temperature control. In this system, a platinum-covered glass capillary, positioned less than 100 μm from the cell under study, was used as a common outlet (52). The pCLAMP 8.1 software (Axon Instruments) was used for acquisition and offline analysis.

Intracellular patch electrochemistry Measurements of cytosolic dopamine concentrations in VM neurons from TH-GFP mice were performed as previously described (32). In brief, a polyethylene-coated 5-μm carbon fiber electrode was placed inside the glass patch pipette and used in a cyclic voltammetric mode of detection (scan rate of 250 mV/ms) using a subroutine locally written in Igor Pro (WaveMetrics Inc.). After achieving a seal between the cell and the patch pipette, the plasma membrane was ruptured by suction, and substances diffusing from the cytosol into the pipette were observed as a slow wave of oxidation current. The cultures were incubated with 100 μM L-DOPA for 1 hour prior to and during the recordings. After background current subtraction, dopamine concentration at the pipette tip was calculated using calibration curves generated for carbon fiber electrodes with different exposed surfaces (34). The initial concentration of cytosolic dopamine was calculated using the neuronal cell body volume and the volume of the pipette tip estimated from photographs acquired before each recording. As the volume of the organelles that occupy neuronal cytosol is unknown, cytosolic dopamine concentrations estimated from intracellular patch electrochemistry recordings were most likely underestimated.

Total cellular levels of catechol were quantified by high-performance liquid chromatography with an electrochemical detection method by comparing to known amount of standards as described before (20, 34, 36).

Isolation of lysosomes Rat liver lysosomes were isolated from a light mitochondrial-lysosomal fraction in a discontinuous metrizamide density gradient by the shorter method described previously (23). Lysosomal integrity was verified after isolation by measuring the activity of β-hexosaminidase, a lysosomal enzyme, in the incubation medium (38). Preparations with more than 10% broken lysosomes immediately after isolation or more than 20% at the end of the incubation were discarded. A lysosomal-enriched fraction was isolated from cultured cells by differential centrifugation as described previously (38).

Promoter luciferase assays.

- A firefly luciferase reporter plasmid containing 3.6 kb of the Tnfrsf11b proximal promoter (pOPG 3.6-luc) was a gift of G. Karsenty, Columbia University (76). An expression plasmid for constitutively active NFATc1 (pMSCV-caNfatc1) was a gift of N. Clipstone, Northwestern University, Chicago, Illinois, USA (77). 293T cells were transiently transfected using the Effectene Reagent (Qiagen) with the indicated luciferase reporter plasmids and a Renilla luciferase vector (pRL-TK) as an internal control at a ratio of 10:1, along with the indicated amounts of pMSCV-caNfatc1. The total amount of DNA per transfection was held constant by supplementing with empty vector DNA. Forty hours after transfection Firefly and Renilla luciferase activities were determined in cell

lysates using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to Renilla luciferase to control for transfection efficiency.

Analysis of cell divisions *in vivo*.

- Purified T cells from DO11.10 and DO11.10p27⁻ mice (10×10^6 cells/ml) were labeled for 30 min at 37 °C with the intracellular fluorescent dye CFSE (5 μM 5(and 6)-carboxyfluorescein succunimidyl ester; Molecular Probes). Then, cells were washed twice with cold RPMI 1640 medium containing 10% FCS, were resuspended in PBS and were transferred intravenously into BALB/c mice (5×10^6 cells per mouse). Syngeneic hosts were left untreated (naive) or were treated with PBS followed by immunization with OVA(323–339) (primed) or with CTLA-4–Ig plus mAb to CD40L followed by immunization with OVA(323–339) as described above (tolerized). Then, 3 d later, lymphocytes were isolated from the draining lymph nodes of the BALB/c hosts. The number of cell divisions on CFSE-stained cells and the percentage of cells that had undergone a specific number of divisions were determined as described[43]. Cells were also stained with mAb KJ1-26 and CFSE analysis of KJ1-26$^+$ T cells was done by flow cytometry.

Adenovirus vectors.

- The cDNA encoding Ras61L was provided by F. Fitch (University of Chicago, Chicago, Illinois). The dominant negative Cbl construct was generated by RT-PCR with cDNA from T_H1 clones as a template and the following primers (upper case, restriction enzyme sequences; underlining, Myc tag sequence): 5'-GGGGTACCatggagcagaaactcatctctgaagaggatctggccggcaacgtgaagaaga-3' (forward) and 5'-ATAGTTTAGCGGCCGCtcaatcttgaggagttggtt cacataa-3' (reverse). The cDNA encoding DGK-α was a gift from M. Topham (University of Utah, Salt Lake City, Utah) and was used as a template to introduce an N-terminal Myc epitope tag by PCR. The sequences of all PCR products were confirmed before subcloning. Construction of recombinant adenovirus vectors was done with a two-cosmid system that has been described[42].

Adenoviral transduction of CAR T cells.

- T_H1 clones were purified from passage cultures by Ficoll-Hypaque centrifugation. Primary CAR 2C *Rag2*$^{-/-}$ CD8$^+$ T cells were isolated from splenocytes by negative selection with magnetic beads and antibody 'cocktails' (Stem Cell Technologies). CAR T_H1 cells were transduced with adenovirus vectors at high cell density (1×10^7 cells/ml) in DMEM containing 2% (volume/volume) FCS and were incubated for 1 h at 37 °C, followed by an overnight 'rest' at 37 °C in DMEM containing 5% (volume/volume) FCS at low cell density (4×10^5 cells/ml).

Lentivirus production and infection protocols.

- A third-generation lentiviral vector encoding EGFP expressed from the human phosphoglycerate kinase promoter was used as described[29, 33]. Cell populations were

incubated overnight (about 16 h) in X-VIVO-10 medium (BioWhittaker) supplemented with 1% BSA (Stem Cell Technologies) and L-glutamine (Invitrogen) with viral supernatant (multiplicity of infection of 130–180). Viral concentrations of 1.0 $\times 10^8$ to 1.8 $\times 10^8$ viral particles/ml, 2.0 $\times 10^7$ to 4.4 $\times 10^7$ viral particles/ml and 0.9 $\times 10^8$ to 1.6 $\times 10^8$ viral particles/ml and cell concentrations of 0.7 $\times 10^6$ to 1.1 $\times 10^6$ cells/ml, 1.0 $\times 10^5$ to 2.5 $\times 10^5$ cells/ml and 0.7 $\times 10^6$ to 1.4 $\times 10^6$ cells/ml for $CD34^+CD38^{lo}$, $CD34^+CD38^-$ and Lin^- cord blood, respectively, were maintained. The efficiency of gene transfer was estimated by progenitor cell assay as described[33].

Lentiviral-mediated RNAi in VM neuron cultures RNAi against LAMP-2A was performed using the shRNAi against the 5′-CTGCAATCTGATTGATTA-3′ sequence of the 1331–1359 bases in exon 8A of the *Lamp-2* gene. The hairpin of this sequence along with the H1 promoter was subcloned from the pSuper vector (described in ref. 13) into the pCCL.PPT.hPGK.GFP.Wpre vector (48). Vesicular stomatitis virus–pseudotyped lentiviral stocks were prepared by transient cotransfection of the shRNAi-carrying vector or empty vector along with the third-generation packaging constructs pMDLg/pRRE, pRSV-REV, and the pMD2.G envelope into 293T cells, as described (48). For transduction, VM-cultured neurons were incubated with 1×10^7 transducing units of concentrated lentivirus at 37°C for 24 hours. After a 1:1 dilution, cells were incubated for another 48 hours and then changed to virus-free fresh medium. The efficiency of RNAi was verified by real-time PCR, immunoblot, and immunofluorescence with an antibody against LAMP-2A at different times. Due to the long half-life of the LAMP-2A protein, we estimated that an 80% decrease in intracellular levels of the protein would not be attained until 5 days after infection. Consequently, the different treatments were all applied after that day. VM cultures infected with the shRNAi or the empty carrying vector were incubated with 100 μM L-DOPA for 48 hours, and the number of neurons remaining after each treatment was calculated after fixation using an Axiovert 200 (Zeiss) fluorescence microscope as described before (30). At least 10 randomly chosen observation fields for each sample were counted from 2 independent samples of each condition.

Electron microscopy and immunogold Immunogold labeling was performed as described previously (23) using an antibody against α-syn for 12 hours, followed by gold secondary (GAR 1:100) for 2 hours. Samples were water rinsed and negatively stained with 1% uranyl acetate. Appropriate controls using only the gold-conjugated secondary antibodies were included. All grids were viewed on a JEOL 100CX II transmission electron microscope at 80 kV.

Measurement of metabolites and hormones. Blood glucose levels were measured using a glucometer (Roche Diagnostics). Serum levels of insulin (Linco Research) and leptin (BioVendor) were determined with murine ELISA kits. Total serum triglycerides (Biomérieux) and FFAs (Roche Diagnostics) were assayed using enzymatic methods. Plasma H_2O_2 levels were measured using an Amplex Red hydrogen peroxide assay kit (Invitrogen).

Transmission electron microscopy. Gastrocnemius muscle was cut into small pieces and fixed in 2% glutaraldehyde for 2 hours at 4° C, postfixed in 1% Osmium tetroxide for 1 hour at 4° C, dehydrated, and embedded in Epon at either a

longitudinal or transverse orientation. The tissue was then cut using an RMC/MTX ultramicrotome (Elexience), and ultrathin sections (60 – 80 nm) were mounted on copper grids, contrasted with 8% uranyl acetate and lead citrate, and observed with a Jeol 1200 EX transmission electron microscope (Jeol LTD) equipped with a MegaView II high-resolution transmission electron microscopy camera. The analysis was performed with Soft Imaging System (Eloïse SARL). The selection of oxidative fibers was based on the size of fibers and the amount of mitochondria.

Mitochondrial DNA analysis. Total DNA was extracted from muscle using phenol/chloroform/isoamyl alcohol (25:24:1) followed by ethanol precipitation. The content of mtDNA was calculated using real-time quantitative PCR by measuring the threshold cycle ratio (ΔCt) of a mitochondrial-encoded gene (COX1, forward 5′ -ACTATACTACTACTAACAGACCG-3′ , reverse 5′ -GGTTCTTTTTTTCCGGAGTA-3′) versus a nuclear-encoded gene (cyclophilin A, forward 5′ -ACACGCCATAATGGCACTGG-3′ , reverse 5′ -CAGTCTTGGCAGTGCAGAT-3′).

Real-time quantitative RT-PCR analysis. Total RNA was extracted with the TRIZOL Reagent (Invitrogen). The level of target mRNAs was measured by RT followed by real-time PCR using a LightCycler (Roche). A standard curve was systematically generated with 8 different amounts of purified target cDNA, and each assay was performed in duplicate (34). Briefly, first-strand cDNAs were first synthesised from 500 ng total RNA in the presence of 100 U Superscript II (Invitrogen) using random hexamers and oilgo(dT) primers (Promega). The real-time RT-PCR was performed in a final concentration of 20 μl containing 5 μl RT reaction medium at 60-fold dilution, 15 μl reaction buffer from the FastStart DNA Master SYBR Green kit (Roche), and 10.5 pmol specific forward and reverse primers (Eurobio). Primer sequences are shown in Supplemental Table 2.

Measurement of mitochondrial respiration on skinned fiber preparation. Mitochondrial respiration was studied in saponin-skinned fibers (35). Fiber bundles were mechanically separated with tongs and permeabilized with saponin (60 mg/l, 20 minutes). Bundles were then washed 3 times for 10 minutes to remove ADP, creatine phosphate, soluble enzymes, and metabolites. Fiber respiration rates were measured at 25° C using an oxygraph system (Hansatech Instruments). Different substrates were used as follows: 5 mM glutamate plus 2 mM malate as complex 1 substrates, 5 mM succinate plus 2.2 μM rotenone as complex 2 substrates with inhibition of complex 1 by rotenone, octanoyl-carnitine (110 μM) or palmitoyl-carnitine (55 μM) in the presence of 1 mM malate as β-oxidation substrates. State 3 was measured in the presence of respiratory substrates after the addition of 1 mM ADP, and state 4 was measured after the addition of 60 μM atractyloside — a potent inhibitor of the ATP/ADP carrier. State 4 was considered the control state of respiration. Finally, fibers were dried for 24 hours at 100° C and weighed. Respiration rates were expressed as nanoatoms

O/(min.mg dried fiber). The respiratory control ratio was calculated by dividing state 3 by state 4 respiration rates.

Mitochondria isolation. Muscle was thawed in isolation buffer (210 mM mannitol, 70 mM saccharose, 50 mM Tris, 10 mM EDTA, and 0.5% BSA; pH 7.4) and cut in small pieces. It was then digested for 15 minutes with trypsin, under agitation, and washed 2 times with the isolation buffer. After each wash, the tissue was centrifuged at 4° C for 2 minutes at 70 *g*. The tissue was homogenized with a conical glass grinder (VWR International) in 1 ml isolation buffer. The homogenate was centrifuged at 4° C for 10 minutes at 820 *g*. The supernatant was then centrifuged at 4° C for 20 minutes at 6,800 *g*. The pellet was resuspended in 1 ml of suspension buffer (225 mM mannitol, 75 mM saccharose, 10 mM Tris, and 0.1 mM EDTA; pH 7.4) and centrifuged at 4° C for 10 minutes at 820 *g*. The mitochondria were then pelleted by centrifuging the supernatant at 4° C for 20 minutes at 6,800 *g* and were resuspended in 50 μl of the same buffer. Western blot analysis was used to determine the cytochrome *c* (1:100; Santa Cruz) content in cytosolic and mitochondrial fractions.

Measurement of enzyme activities. CS activity was measured spectrophotometrically (36) in purified mitochondria from gastrocnemius muscle. For the measurement of caspase 3 activity, gastrocnemius muscle was lysed in ice-cold lysis buffer (1% NP-40, 20 mM Tris-HCl, 138 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5% glycerol; pH 8.0) and supplemented with 5 mM EDTA (1 mM Na_3VO_4, 20 mM NaF, 1 mM DTT, and protease inhibitors). After centrifugation at 10,000 *g* for 10 minutes at 4° C, aliquots of supernatant (100 μg) were incubated with 10 μl DEVD-pNA for 1 hour at 37° C according to the instructions of the manufacturer (Clinisciences). The pNA light emission was quantified using a spectrophotometer at 400 nm.

Protein carbonylation. The Oxyblot Oxidized Protein Detection Kit was purchased from Chemicon. The carbonyl groups in the protein side chains were derivatized to DNP-hydrazone by reaction with DNPH following the manufacturer's instructions. After the derivatization of the protein sample, 1-dimensional electrophoresis was carried out on a 10% SDS-PAGE gel. Proteins were transferred to PVDF membranes. After incubation with anti-DNP antibody, the blot was developed using a chemiluminescence detection system.

Muscle cell culture. C_2C_{12} myoblasts were grown to confluence in DMEM supplemented with 10% FCS. They were induced to differentiate into myotubes by switching to DMEM containing 2% horse serum. Primary cultures of human skeletal muscle cells were initiated from satellite cells of quadriceps samples obtained from 3 male organ donors (age, 27 ± 7 years; body mass index, 23 ± 1.7 kg/m^2). Differentiated myotubes were prepared according to the procedure previously described in detail (37). Myotubes were then cultured for 96 hours, with or without H_2O_2 (100 μM),

with mannitol (25 mM) or glucose (25 mM), and with BSA (1%) or palmitic acid (200 μM), in the presence or absence of NAC (10 mM).

ROS production in C_2C_{12} cells. ROS production was detected using the nitroblue tetrazolium (NBT; Sigma-Aldrich) assay. NBT is reduced by ROS to a dark-blue insoluble form of NBT called formazan. After treatment, myotubes were incubated for 90 minutes in DMEM containing 0.2% NBT. Formazan was dissolved in 50% acetic acid, and the absorbance was determined at 560 nm. Optical density values were normalized by protein levels.

In vitro macrophage culture. Peritoneal macrophage cultures were isolated 5–6 days after 3% thioglycollate injection. The peritoneum was washed, and macrophages were cultured overnight. Macrophages (75%–94% CD3-$CD11b^+F4/80^+$) were infected with *L. major* promastigotes (10 parasites/macrophage). Five hours after infection, 3 μg CpG 1826 was added to the cultures; 24 hours after infection, supernatants were removed and mRNA extracted from macrophages for real-time RT-PCR analysis.

Cell purification. Single cell suspensions were isolated from retromaxillary LNs and ear tissue. Ears were split into dorsal and ventral sides and incubated in 1 mg/ml dispase/collagenase (Roche) for 30 minutes at 37° C. Cells were cultured in complete RPMI 1640 with 10% heat-inactivated FCS. T cells were depleted from single cell suspensions of APCs via positive selection using magnetically assisted chemical separation for $Thy1.2^+$ cells.

In vitro cell culture and cytokine analysis. Single-cell suspensions from LN and ear tissue were cultured on anti-cytokine mAb-coated plates in the presence or absence of SLA (equivalent to 2×10^6 organisms/ml) or 5 μM OVA peptide 323–339 for 6 hours for IL-4 and IFN-γ measurement by ELISPOT as described previously (62). Control uninfected ears processed in the same way routinely yielded <10 cytokine-producing spots per ear.

For ex vivo cytokine analysis, the Cytokine Capture Assay (Miltenyi) was performed essentially as described previously (62). Single cell suspensions were isolated directly ex vivo from the ear 2 weeks after infection with *L. major*. Cells were labeled with the bifunctional Ab "catch" reagent for 5 min on ice and warmed to 37° C with SLA for 45 minutes for cytokine secretion. Cytokine was detected by FACS using a second anti-cytokine mAb. Gates were drawn on cells labeled without the catch reagent ("no catch"). Th1 and Th2 effectors were generated from DO11.10 TCR Tg^+ CD4 T cells in vitro as described previously (62).

For cytokine analysis after in vitro culture, LN and ear cell suspensions (10^7/ml) were incubated with 5 μM CFSE (Molecular Probes) for 5 min at room temperature and washed 3 times before culture. Cells were incubated for 72 hours at 37° C with or without exogenous SLA. Intracellular cytokine staining was performed using a BD Pharmingen kit. Brefeldin A was added to the cultures 4 hours before harvest.

Chemokine analysis. Ears were surgically removed, placed on ice, and homogenized in TRIZOL (Invitrogen) for extraction of mRNA and then reverse-transcribed (RT for PCR kit; BD Clontech). Real-time RT-PCR used Assays-on-Demand Taqman primer/probe sets with an ABI prism 7900 sequence detection system (Applied Biosystems). Target levels were normalized to HPRT.

Apoptosis induction.

- Spontaneous apoptosis of PMNs was detected after 22 h of incubation in culture media. In some experiments, zVAD-fmk (10-50 μM), TNF (40 ng/ml), resolvin E1–methyl ester, aspirin-triggered lipoxin A_4 analog, PD1–methyl ester (10 nM) or TGF-β (10 ng/ml) was added. Vehicle treatment was 0.05% (volume/volume) ethanol. Peripheral blood T cells were activated by incubation for 3 d in 24-well plates coated with anti-CD3 (5 μg/ml; R&D Systems). Jurkat cells or activated peripheral blood T cells were incubated for 4–48 h with staurosporine (1–2 μM) or Fas ligand (0.05–5 ng/ml), after which cells were collected and used for flow cytometry or binding assays. In some experiments, zVAD-fmk (10–50 μM; R&D Systems) was added to cells 20 min before the addition of apoptosis-indu

Terminal deoxynucleotidyl transferase 2'-deoxyuridine 5'-triphosphate nick end labeling assay. Apoptotic cells were detected with an ApopTag apoptosis detection kit (Invitrogen) following the manufacturer's protocol. Propidium iodide staining was performed by incubating sections with propidium iodide (20 μg/ml) for 10 minutes at room temperature. Cisplatin (5 μg/ml) was used as a positive control to induce apoptosis.

TUNEL staining. Epo-treated (i.p. 5,000 U/kg body weight, P6, P8, P10, and P12) or control P17 mice were anesthetized, and eyes were enucleated and fixed in 4% paraformaldehyde (1 h). Tissue was then cryoprotected in 30% sucrose overnight, embedded in OCT (Fisher Scientific Co.), and sectioned with a cryostat at 14 μm. Sequential vertical sections cut through the center of the eye were labeled by TUNEL with Roche in situ cell death detection kit (catalog no. 11684795910; Roche Applied Science) followed by DAPI staining (catalog no. H1200; Vector Laboratory). DAPI staining was displayed with a red LUT to better visualize colocalization with TUNEL staining (green fluorescence). Retinas were examined using fluorescence microscope, and TUNEL-positive fluorescent intensity was scored.

TUNEL assay. Paraffin-embedded sections were stained with In situ Apoptosis Detection Kit (TaKaRa Bio Inc.), according to the manufacturer's instructions, to detect apoptotic cells. TUNEL-positive cells were counted on 5 randomly chosen visual fields at ×400 magnification.

Caspase activity assay. Epo-treated (i.p. 5,000 U/kg body weight, P6, P8, P10, and P12) or control P17 mice were anesthetized, and retinas were isolated and homogenized in Glow Lysis Buffer (catalog no. E2661; Promega). Retinal homogenate (50 μg) was analyzed for caspase-3/-7 activity using Promega Caspase-Glo 3/7 Assay system (G8091; Promega).

NF-κB activity analysis. NF-κB–Luc reporter mouse littermates were given PBS or Epo treatment (i.p. 5,000 U/kg body weight) on P6 and P7. After 18 h oxygen exposure, P8 pups were sacrificed and retinas were isolated and homogenized in Glow Lysis Buffer (catalog no. E2661; Promega). Retinal homogenate (50 μg) was analyzed for luciferase activity using Promega Bright-Glo Luciferase Assay system (catalog no. E2610). Additionally, retinal cross-section of NF-κB–Luc reporter mouse were taken at P8 and stained with rabbit antibodies to luciferase (catalog no. ab21176; Abcam).

Mice strains and genotyping.

- The 129/Sv *Rhoh*$^{-/-}$ mice were generated by Targeting Laboratory. The entire coding region of mouse *Rhoh* is in its third exon; the targeting vector was designed to replace the third exon of *Rhoh* with a neomycin-resistance cassette. The genotypes of *Rhoh* gene-targeted embryonic stem cells and transgenic mice were determined by Southern blot analysis of DNA digested with *Spe*I using a 5' *Rhoh* genomic DNA probe or by PCR analysis with primers. The 129/Sv *Rhoh*$^{-/-}$ mice were crossed with wild-type or p14 TCR (V_α2Vβ8) transgenic mice on a C57BL/6J background to generate *Rhoh*$^{-/-}$ or p14$^{tg/+}$*Rhoh*$^{-/-}$ compound mice. Mice used were littermates derived from backcross generations with an N of more than 2. The 129S6/SvEvTac-*Rag2*$^{-/-}$ mice were purchased from Taconic Animal Models. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Cincinnati Children's Hospital Research Foundation (Cincinnati, Ohio).

Antibodies and GST fusion proteins.

- Fluorescence-conjugated monoclonal antibodies to the following mouse antigens were used for flow cytometry: CD4 (RM4-5), CD8α (53-6.7), CD25 (7D4), CD44 (IM7), TCR β-chain (H57-597), TCR γδ (GL3), TCR Vβ8, TCR Vβ5 (MR9-4), CD69 (H1.2F3), CD5 (53-7.3), Gr-1 (RB6-8C5), Mac-1 (M1-70), NK1.1 (PK136), Thy1.2 (53-2.1), CD45R–B220 (RA3-6B2), IgM (R6-60.2), BrdU (3D4) and Ter119 (Ly-76; all from Pharmingen). For immunoblot analyses, antibodies to the following were used: RhoH[9] (B4998), Zap70 phosphorylated at Y319 (17a), phosphorylated tyrosine (4G10) and Lat (45; Pharmingen); hemagglutinin (3F10; Roche); β-actin (AC-15; Sigma); CD3ζ (6B10.2; Santa Cruz Biotechnology); and Lat phosphorylated at Y191 (3584), Zap70 (99F2), phosphorylated p42-p44 (Thr202-Tyr204; 197G2) and p42-p44 (9102; Cell Signaling Technology). Primary antibodies were detected with the secondary antibodies horseradish peroxidase–conjugated goat anti-mouse (7076) or goat anti-rabbit (7074; both Cell Signaling Technology), or donkey anti-rat (sc-2956; Santa Cruz Biotechnology) using enhanced chemiluminescence detection (Cell Signaling Technology). GST fusion proteins were expressed in *Escherichia coli* BL21 (DE3) cells and were purified

according to the manufacturer's recommendations (GE Healthcare Life Science). Purified GST fusion protein lysates were incubated for 1 h at 4 °C with glutathione–Sepharose 4B beads. Bead-bound GST fusion proteins were separated by SDS-PAGE and were quantified by Coomassie blue staining.

Rat Brain Preparation and Immunoprecipitation

- Whole brains from rats (Sprague-Dawley) in equal volume of UCH buffer were homogenized using 1.0mm glass beads (Biospec Products) with Mini-Beadbeater (Biospec Products). The lysate was centrifuged at 14,000 rpm for 10 min and the supernatant was manually loaded onto a 1 ml HiTrap Q XL column. The column was then installed onto FPLC system and washed with 5 column volume of UCH buffer. Proteins were eluted with a gradient of 0 to 1 M NaCl in UCH buffer over 60 column volume. A small fraction of synuclein was retained in the column and they were coeluted with UCH-L1 (from 200 to 300 mM NaCl gradient). The fractions that contained UCH-L1 and synuclein were determined by Western blotting. They were combined and dialyzed in UCH buffer at 4_C overnight. For each immunoprecipitation assay, 0.5 ml of combined UCH-L1/synuclein fractions was added with 1 _l of SYN-1, Anti PGP9.5, or buffer. Binding reactions were sat at RT for 1 hr prior to addition of protein G agarose (Boehringer Mannheim). The mixtures were rotated using Labquake Shaker (Barnstead) for half an hour. The agarose was isolated by centrifugation and washed with UCH buffer three times. Proteins were eluted with SDS loading buffer and result was analyzed using Western blot.

Coimmunoprecipitation. HEK293 cells expressing both CLDN16 and CLDN19 were lysed in 50 mM Tris (pH 8.0) by 25–30 repeated passages through a 25-gauge needle, followed by centrifugation at 5,000 *g*. The membranes of lysed cells were extracted using CSK buffer (150 mM NaCl; 1% Triton X-100; 50 mM Tris, pH 8.0; and protease inhibitors). The membrane extract was precleared by incubation with protein A/G-sepharose (Sigma-Aldrich) prior to coimmunoprecipitation. The precleared membrane extract was incubated for 16 h at 4° C with anti-CLDN2, anti-CLDN16, and anti-CLDN19 antibodies. Antibody-bound material was pelleted with protein A/G-sepharose, washed 3 times with CSK buffer, and detected by immunoblotting.

Immunolabeling and confocal microscopy. Cells grown on coverslips were fixed with 4% paraformaldehyde in 0.1 M PBS; cells grown on Transwell inserts (Corning) were fixed with cold methanol at −20° C, followed by blocking with PBS containing 10% FBS and incubation with primary antibodies (diluted 1:300) and FITC- or rhodamine-labeled secondary antibodies (diluted 1:200). After washing with PBS, slides were mounted with Mowiol (CalBiochem). Confocal analyses were performed using the Nikon TE2000 confocal microscopy system equipped with Plan-Neofluar ×40 (NA 1.3 oil) and ×63 (NA 1.4 oil) objectives and krypton-argon laser (488 and 543 lines). For the dual imaging of FITC and

rhodamine, fluorescent images were collected by exciting the fluorophores at 488 nm (FITC) and 543 nm (rhodamine) with argon and HeNe lasers, respectively. Emissions from FITC and rhodamine were detected with the band-pass FITC filter set of 500 - 550 nm and the long-pass rhodamine filter set of 560 nm, respectively. All images were converted to JPEG format and arranged using Photoshop 6.0 (Adobe).

Freeze fracture. Confluent monolayers of mouse L cells individually expressing or coexpressing CLDN16 and CLDN19, or confluent monolayers in which L cells expressing CLDN16 were cocultured with L cells expressing CLDN19, were fixed in 2% glutaraldehyde in Dulbecco PBS (DPBS) for 20 minutes at 4° C. They were rinsed twice in DPBS, scraped from the substrate, and infiltrated with 10%, 20%, and 25% glycerol in 0.1 M cacodylate buffer, pH 7.3, over 1 h at 4° C. Cell pellets were frozen in liquid nitrogen slush and freeze fractured at -115° C in a Balzers 400 freeze-fracture unit. After cleaning with sodium hypochlorite, replicas were examined by electron microscopy at a magnification of ×62,500.

Freeze fracture immunolabeling. The method for immunogold labeling was described previously (25). Briefly, the monolayers were fixed in 1% paraformaldehyde in DPBS for 15 minutes at 4° C. They were rinsed 3 times in DPBS, scraped from the substrate, and infiltrated with 10%, 20%, and 25% glycerol in 0.1 M cacodylate buffer over 1 h at 4° C. Cell pellets were frozen in liquid nitrogen slush and freeze fractured at -115° C. The replicas were cleaned by floating them on the surface of 2.5% SDS, 30 mM sucrose in 10 mM Tris-HCl (pH 8.3) using a magnetic stir plate for 2 h at room temperature. The replicas were rinsed in 4 changes of DPBS over 1 h and incubated with 5% ovalbumin in DPBS for 30 min. They were then incubated with rabbit anti-CLDN16 or anti-CLDN19 (diluted 1:100 in blocking solution) for 1 h at room temperature. After replicas were rinsed in DPBS, they were incubated for 1 h with protein A gold (10 nm) diluted 1:100 in ovalbumin/DPBS. After rinsing in DPBS, the labeled replicas were fixed with 0.5% glutaraldehyde in DPBS and picked up on Formvar-coated copper grids. Replicas were examined with a Philips 301 electron microscope.

Electrophysiological measurements. Electrophysiological studies were performed on cell monolayers grown on porous filters (Transwell) as previously described (4). Voltage and current clamps were performed using the EVC4000 Precision V/I Clamp (World Precision Instruments) with Ag/AgCl electrodes and an agarose bridge containing 3 M KCl. TER was measured using the Millicell-ERS and chopstick electrodes (Millipore). TER of the confluent monolayer of cells was determined in buffer A (145 mM NaCl, 2 mM $CaCl_2$, 1mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4), and the TER of blank filters was subtracted. Dilution potentials were measured when buffer B (80 mM NaCl, 130 mM mannitol, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4) replaced buffer A on the apical or basal side of filters. Electrical potentials obtained from blank inserts were subtracted from those obtained from inserts with confluent growth of cells. P_{Na}/P_{Cl} for the monolayer was calculated from the dilution

potential using the Goldman-Hodgkin-Katz equation. P_{Na} and P_{Cl} were calculated using the Kimizuka-Koketsu equation. P_{Mg} across monolayers was determined as described previously (4).

Inhibition of galactosyltransferase. BADG was used as inhibitor of β1,3-galactosyltransferase (36). IgA1-secreting cell lines (1×10^6 cells) from IgAN patients and controls were cultured with or without 5 mM BADG dissolved with 3.7% methanol in RPMI 1640 medium supplemented with L-glutamine, 20% FCS, penicillin, and streptomycin. After 72 hours, levels of IgA1 and HAA-reactive IgA1 produced by the cell lines were measured by ELISA.

In situ hybridization. Sense and antisense digoxigenin-labeled probes were transcribed from a linear pCR4Blunt-TOPO (Invitrogen) plasmid carrying *Eya4* exons 8 to 12 cDNA. An 855-bp probe for *Six1* was amplified using the following primers: *Six1*, forward, ATGTCGATGCTGCCGTCGTTTGG; and *Six1*, reverse, TTAGGAACCCAAGTCCACCAAACTGG. A 720-bp probe for *Eya1* was amplified using the following primers: *Eya1*, forward, ATGGAAATGCAGGATCTAACCAGC; and *Eya1*, reverse, CGTCATGTAGTGTGCTGGATAC. Fragments were cloned into pCR4Blunt-TOPO (Invitrogen) plasmid, transcribed as described for *Eya4* probes, and hybridized to either whole mouse E12.5 embryos or to 10-μm paraffin-embedded sections (44).

In vivo cross-linking and coimmunoprecipitation. To examine association between ARHI and LC3 in vivo, a cell membrane–permeable cross-linker, BASED (Bis-[β-(4-azidosalicylamido) ethyl] disulfide) (Pierce Biotechnology) was used. Cells were rinsed twice in cold PBS and treated with 5 mM BASED for 20 minutes before being exposed to UV light (366 nm). Cells were lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS), supplemented with protease inhibitor cocktail (Roche Applied Science) and 1 mM PMSF. Coimmunoprecipitation and western blotting were performed as described in the figure legends.

Capillary electrophoresis

- High-performance capillary electrophoresis (HPCE) was performed on a Beckman system P/ACE 2100. An untreated capillary (fused silica, 57 cm total length x 75 μ m I.D.) was used, protein samples were injected by pressure and detection was performed by measuring UV absorption at 200 nm. Runs were carried out in 0.1 M sodium phosphate buffer (pH=2.0) containing 0.02% HPMC at a constant voltage (12 kV) and at a capillary temperature of 25°C (40-43).

Enzymatic Cleavage

- Histone H1t subfractions (~100 μg) obtained by HILIC fractionation were digested either with α -chymotrypsin [EC 3.4.21.1] (Sigma type I-S, 1/150 w/w) in 100 μl of 100 mM sodium acetate buffer (pH=5.0) for 30 min at room temperature or with Endoproteinase Arg-C [EC 3.4.21.1] (Roche, sequencing grade, 1/20 w/w) in 100 μl of 90 mM Tris-HCl, 8.5mM CaCl2 buffer (pH=7,6) containing 5mM DTT/0.5mM EDTA activator for 20 min at room temperature. The digests were subjected to LC-MS.

Hydrophilic Interaction Liquid Chromatography

- The histone fraction H1t (~200 µg) isolated from rat and mouse testis by RP-HPLC was analyzed on a PolyCAT A column (100 mm x 4.6 mm I.D.; 3 µm particle size; 150 nm pore size; PolyLC) at 18°C and at a constant flow of 1.0 ml/min using a two-step gradient starting at solvent A - solvent B (100:0) (solvent A: 70% acetonitrile, 0.015 M TEA/H3PO4, pH=3.0; solvent B: 70% acetonitrile, 0.015 M TEA/H3PO4, pH=3.0 and 0.68 M NaClO4). The concentration of solvent B was increased from 0% to 60% B during 5 min, from 60% to 100% during 35 min, and then maintained at 100% during 30 min.

High-performance Liquid Chromatography

- The equipment used consisted of a 127 Solvent Module and a Model 166 u.v.-visible-region detector (Beckman Instruments, Palo Alto, CA, USA). The effluent was monitored at 210nm and the peaks were recorded using a Beckman System Gold software.

Reversed-phase HPLC

- The separation of whole linker histones was performed according to Lindner et al. (36-39) using a Nucleosil 300-5 C4 column (250 mm x 8 mm I.D.; 5 µm particle pore size; 30 nm pore size; end-capped; Machery-Nagel). The lyophilized proteins were dissolved in water containing 200 mM 2-mercaptoethanol, and samples of ~400 µg were injected onto the column. The histone H1 sample was chromatographed within 40 min at a constant flow of 1.5 ml/min with a two-step acetonitrile gradient starting at solvent A - solvent B (63:35) (solvent A: water containing 0.1% TFA and 15% EGME; solvent B: 70% acetonitrile, 0.1% TFA and 15% EGME). The concentration of solvent B was increased from 35% to 58% B during 35 min, hold for 5 min at 58% B, and increased from 58% to 100% B during 8 min.

Mass-spectrometric Analysis

- Arg-C digests of the fractions H1tp0-p3 were analysed using nano-HPLC consisting of an UltiMate 3000 system (Dionex Corporation) connected online to a linear iontrap mass spectrometer (ThermoElectron Finnigan LTQ) equipped with a nanospray ionization source. The nanospray voltage was set at 1.6 kV and the heated capillary was held at 200°C. Data-dependent neutral loss MS3 and multistage activation were used for precise localization of phosphorylation. MS/MS and MS3 spectra were searched against a histone database using SEQUEST (LCQ BioWorks; ThermoFinnigan) and validated manually. The identified peptides were further evaluated using charge state versus cross-correlation number (Xcorr). The criteria for positive identification of peptides were Xcorr > 1.5 for singly charged ions, Xcorr > 2.0 for doubly charge ions, and Xcorr > 2.5 for triply charge ions. Only best matches were considered. MS/MS tolerances of + 1Da were allowed.

GST precipitation assay.

- Jurkat cells were lysed in GST lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1% Nonidet-P40 and Complete Protease Inhibitors). Cell lysates were loaded onto columns of bead-bound GST fusion proteins. After columns were washed with GST lysis buffer containing 150 mM and 200 mM NaCl, bound proteins were eluted with GST

lysis buffer containing 400 mM NaCl and SDS sample buffer, sequentially. Eluted proteins were detected by SDS-PAGE and Coomassie blue staining. Protein bands were identified with a Bruker Biflex III MALDI-TOF mass spectrometer (SpectroREADER; Sequenom) and Protein Mass Fingerprinting Mascot search (Matrix Science).

Subcellular fractionation.

- Cells were lysed by brief sonication on ice in a buffer of 250 mM sucrose, 20 mM Tris, pH 7.8, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM Na_3VO_4, 10 mM NaF and Complete Protease Inhibitors. Lysates were centrifuged to remove nuclei and debris (900*g* for 5 min at 4 °C). The P100 and S100 fractions were separated by centrifugation for 30 min at 100,000*g*. Membrane fractions were made soluble with MLB (Upstate) plus protease and phosphatase inhibitors. After centrifugation for additional 30 min at 100,000*g*, the detergent-insoluble cytoskeleton-containing fraction was resolved by 0.5% SDS-PAGE.

Assessment of Intracellular Calcium Concentration

- Jurkat cells cultured in calcium-free RPMI-1640 medium (Gibco BRL; number 22300-107) containing calcium-free 10% FBS were triggered by anti-Fas IgM. The treated cells were harvested at the indicated time points and incubated with Fluo-3-AM at a final concentration of 1 micromolar for 30 min at 37°C (Scoltock et al., 2000). The labeled cells (50,000 cells per treatment) were then analyzed by exciting the cells at 488 nm and examining the fluorescence emission of Fluo 3 at 530 nm with a FACS Scan, (Becton Dickinson). A one micromolar concentration of LPA was used as a positive control for Ca2+ induction. The data thus obtained was analyzed with the software Win MDI 2.8 and represented as contour plots.The effect of chelating intracellular calcium on translocation of annexin I was studied by culturing Jurkat cells in the presence of 10 micromolar BAPTA-AM, with or without the addition of anti-Fas IgM. Cells were harvested and fractionated as detailed above, and the S-100 fractions were assessed by immunoblotting for presence or absence of annexin I.

Mouse bone marrow transduction and transplantation.

- Retrovirus-mediated transduction of mouse bone marrow cells was done by published methods[49]. Prestimulated low-density bone marrow cells were infected with high-titer retrovirus supernatant on fibronectin-coated plates. Retrovirus supernatant was generated in the phoenix-gp cells with a mouse stem cell virus–based retroviral vector coexpressing EGFP and HA–RhoH as described[50]. $EGFP^+$ sorted cells were transplanted by intravenous injection into the sublethally irradiated (300 rads with a ^{137}Cs irradiator) *Rag2*$^{-/-}$ recipient mice. At 9 weeks after transplantation, thymus, peripheral blood, bone marrow, spleen and lymph nodes from each recipient mouse were collected for analysis of $EGFP^+$ chimerism and hematopoietic lineage by flow cytometry. Expression of HA–RhoH and HA–RhoHF73F83 in $EGFP^+$ sorted thymocytes of recipient mice was confirmed by immunoblot analysis.

Determination of renal morphology

- Kidney slices were postfixed in buffered 2% OsO4, dehydrated, and embedded in an Araldite-EM bed 812 mixture. Large sections were cut perpendicular to the renal capsule, containing cortex, and medulla. Thin (1 μm) sections were analyzed in a blinded manner for morphologic alterations, as previously detailed

Two-photon microscopy.

Pregnant *Cx3cr1*^{gfp} mice (18) were i.v. injected with 200 μl of 0.5 mg/ml TRITC-dextran (150 kDa; Sigma-Aldrich) that was allowed to circulate for 3–5 minutes prior to sacrifice to visualize blood vessels. Uterine tissue was retrieved and immediately visualized by 2-photon microscopy. Two-photon imaging was performed with a Zeiss LSM 510 META NLO microscope equipped with ×20 and ×40 water immersion objectives. A Mai Tai One Box Ti:Sapphire Laser (Spectra Physics, Newport Corp.) was used for 2-photon excitation. Image acquisition was performed using LSM 510 acquisition software. For 2-photon excitation of TRITC-dextran, the laser was tuned to 800 nm, and the emission filter set was 535–590 nm. For excitation of GFP, the laser was tuned to 920 nm, and the emission filter was 500–550 nm.

NMR.

Tumors were collected 30 minutes after [3-^{13}C]lactate delivery to mice, weighted, washed with cold PBS, and extracted with cold 0.9 M perchloric acid. Tumor extracts were neutralized and lyophilized. ^{13}C NMR analysis was carried out with a 500-MHz Varian Inova spectrometer operating at 125.7 MHz, with 45° pulse and 0.8-second repetition time in a 5-mm tunable broadband probe. Dioxane was used as an internal standard.

In vivo contrast-enhanced MRI.

MRI was acquired at 4.7T (Bruker) using a whole-body coil. A series of variable-flip-angle precontrast T1-weighted 3D gradient-echo images was acquired to determine the endogenous precontrast R1 (pulse flip angle, 15°, 5°, 30°, 50°, 70°; repetition time [TR], 10 ms; time to echo [TE], 3.6 ms; 2 averages; spectral width, 50,000 Hz; 4 × 4 × 4 cm matrix size, 128 × 128 × 64, zero-filled to 128 × 128 × 128, resulting in a voxel resolution of 313 × 313 × 313 μm [625 μm is the resolution in the third dimension before zero-filling]; total acquisition time per frame, 164 seconds; frequency-encoding direction: head-foot). For dynamic contrast-enhanced MRI, 9 mg/mouse (20 g) in 0.2 ml PBS of biotin-BSA-GdDTPA (r = 164 mM^{-1} s^{-1}) was administered via a tail vein catheter and its extravasation monitored over time (MR data were acquired with a pulse flip angle of 15°, and all other parameters as stated above. The first image was acquired 0–164 seconds after administration of the contrast material.) The MRI data allowed quantification of the fBV and the PS (rate of extravasation of contrast material to the interstitial space) on ISs of E5.5, as previously reported (28). In brief, the change in the concentration of the

administered MRI contrast material (biotin-BSA-GdDTPA) over time (*Ct*) in the region of interest of the ISs was divided by its concentration in blood (*Cblood*; calculated in the region of interest depicting the vena cava, which was included in the image data set, and extrapolated to time 0). Linear regression of these temporal changes in *Ct*/*Cblood* yielded 2 parameters (the fBV and PS) that characterize vascular development: fBV (= *C0*/*Cblood*) was derived from the concentration of biotin-BSA-GdDTPA in the tissue (*Ct*) extrapolated to the time of administration of the contrast material (*C0*) and divided by its concentration in the vena cava (*Cblood*). *PS* [= (*Ct* − *C0*)/(*Cblood* × *t*)] is the rate of contrast extravasation and accumulation in the tissue derived from the slope of the linear regression of the first 15 minutes after contrast material administration (*t* = 15).

Fluorescence image analysis.

All fluorescence images (of anti‐phospho‐histone H3 and anti-Cx43, as well as avidin-FITC stainings) were acquired under identical conditions and analyzed using ImageJ software (http://rsbweb.nih.gov/ij/). The fluorescence intensity was measured in absolute counts. The average fluorescence intensity inside the region of interest in the IS was calculated by measuring the ratio of fluorescence signal to the area of region of interest. When probing for vessel density and vessel permeability (after avidin-FITC staining of the MRI contrast material), the areas occupied by clearly resolved blood vessels (attributed to the myometrium or to undecidualized uterine stroma) were masked and excluded from calculation. Fluorescent background was subtracted from each image, where background was measured in the areas without tissue. Vessel density was estimated by determining mean fluorescence at 3 minutes after injection. Vessel permeability was simulated by the change in fluorescence signal intensity between 3 and 15 minutes after injections.

pH and metabolite measurements.

Extracellular pH was measured with a pH meter. Intracellular pH was measured at extracellular pH 7.3 using the pH sensor 5-(and-6)-carboxy-seminaphthorhodafluor-acetoxymethylester (C.SNARF1-AM; Invitrogen), as previously described (33). Lactate and glucose concentrations were measured on deproteinized supernatant samples using a CMA600 microdialysis analyzer.

ATP assay. On day 0, tumor cells (10^5 in 200 μl of medium as indicated in Figure 5, A and B) were seeded in 96-well plates. ATP cell content was determined over time using the ATP Bioluminescent Somatic Cell Assay kit (Sigma-Aldrich)

according to the manufacturer's recommendations. The conversion of luciferin to oxoluciferin in the presence of ATP generated a fluorescent signal proportional to intracellular ATP that was measured with an IVIS50 Imaging System (Xenogen Corp.).

Mouse xenograft and measurement of tumor size. Eight-week-old BALB/c nu/nu mice were purchased from Charles River Laboratories. SKOv3-ARHI, SKOv3-NTD, or parental SKOv3 cells (1×10^7) were injected subcutaneously into the flank of each mouse. DOX (2 mg/ml) in 5% glucose or glucose alone was added to the drinking water on the day of injection. For the CQ experiment, DOX-treated mice were injected intraperitoneally with CQ (5 days/week at 50 mg/kg) for the entire 5 weeks or the final 2 weeks of DOX treatment. Tumor size was measured every third day using a digital caliper. The mean of 2 independent measurements was averaged. All procedures were carried out according to the animal protocol approved by the Institutional Animal Care and Use Committee of the M.D. Anderson Cancer Center at the University of Texas.

Antibody arrays. Human and mouse cytokine/growth factor antibody arrays were purchased from RayBiotech, and angiogenesis antibody arrays were from Panomics. SKOv3-ARHI cells were seeded at 80% confluence with 1% FBS and induced by adding DOX in culture medium. Medium and cell lysates were collected after 48 hours. Lysates of cells in vivo were prepared from frozen tissues of isolated xenografts.

VEGF ELISA assay. VEGF ELISA kits were purchased from R & D Systems. SKOv3-ARHI cells were seeded at 80% confluence with 1% FBS. After 24 hours, DOX was added to induce ARHI expression. Medium and cell lysates were collected after an additional 24 hours in the presence or absence of hypoxia (in a hypoxia chamber filled with 1% O_2, 5% CO_2, and 94% N_2).

Measurements of Ca^{2+}_i. Cultures were loaded with fura-2-AM (5 μM at 37° C for 40 minutes) and imaged with a ×40 oil objective on a Nikon TE300 microscope outfitted with an Orca camera (Hamamatsu), and fluorescence was acquired alternately at 340 and 380 nm (emission >450 nm). In all cases, background light was measured and subtracted by exposing cells to digitonin (15 μM) and $MnCl_2$ (10 mM) as previously described (47).

Confocal microscopy. To label ASL, Ringer's solution containing Texas Red dextran was added to HBEC mucosal surfaces. Perfluorocarbon (PFC) was added mucosally to prevent ASL evaporation, and the cultures were imaged using a Leica SP5 confocal microscope with a ×63 glycerol immersion objective. Five points per culture were scanned and an average ASL height determined using ImageJ (http://rsbweb.nih.gov/ij/). Nucleotides were added to mucosal surfaces as dry powders in PFC at approximately 200 μM (2). We accelerated/decelerated the HBECs inside a highly humidified incubator to generate 0.5 dyne/cm^2 apical shear stress at culture surfaces as previously reported (2).

For labeling of plasma membrane HA-$P2Y_2$-R, BHK cells grown on glass coverslips were precooled on ice for 10 minutes before being labeled for 45 minutes with anti-HA monoclonal antibodies in PBS. Cells were washed 3 times with ice-cold PBS to remove the antibodies and then warmed to 37° C and treated with ATP with or without E2. The experiment was stopped at different times by fixing the cultures with 4% PFA (for 10 minutes at 21° C). After washing 3 times in PBS, cells were blocked with 1% BSA and 5% normal goat serum (NGS) before addition of secondary antibody for 1 hour. To quantify HA-$P2Y_2$-R internalization, 10 regions of interest were drawn inside the cytoplasm of each cell, which approximated 10% of total cell area and which excluded the space occupied by the nucleus. The intensity of HA-$P2Y_2$-R was normalized to the area of the region of interest sampled using ImageJ.

Immunohistochemistry and confocal microscopy. Immunohistochemistry and confocal microscopy protocols are available at http://www.med.upenn.edu/mcrc/histology_core/. Primary antibodies utilized for immunohistochemistry studies included the following: monoclonal anti-calponin antibody (hCP) (C2687), monoclonal anti-Cre antibody (clone7-23), monoclonal anti-SMA (1A4) (Sigma-Aldrich), polyclonal anti-SM22α antibody (Ab10135) (Abcam); anti-smooth muscle myosin heavy chain IgG (BT-562) (Biomedical Technologies), polyclonal anti-fibronectin (Cell Signaling), monoclonal anti-GFP (Chemicon), monoclonal anti-GAPDH (Chemicon), and polyclonal anti-laminin (Sigma-Aldrich). Secondary antibodies utilized included the following: Alexa Fluor 488 goat anti-mouse and rabbit anti-mouse, Alexa Fluor 568 goat anti-rabbit and rabbit anti-mouse (Invitrogen), and donkey anti-goat IgG, donkey anti-rabbit IgG, and donkey anti-mouse IgG HRP (Jackson ImmunoResearch). Tissue sections were fixed in paraformaldehyde, and where indicated stained for β-galactosidase activity as previously described (46). TUNEL staining of *Myocd*$^{F/F}$/*Wnt1-Cre*$^{+}$ and *Myocd*$^{F/F}$ control embryos harvested at E12.5, E14.5, E16.5, and P2 was performed as described (49). Confocal microscopy was performed on a Leica PCS SP2 microscope.

Immunohistochemical detection of TNF-α in human colon tissues. The tissues were obtained upon biopsy from patients with UC and colorectal cancer with an informed consent and with approval from the Human Subjects Research Ethical Committee of Kanazawa University Hospital. The tissues were fixed and paraffin-embedded and were cut at 5 μm. Paraffin-embedded sections were additionally deparaffinized for immunohistochemical analysis, using the combination of anti-human TNF-α mouse monoclonal antibody and CSA system.

Bone marrow chimeric mice generation. Cell suspensions from male WT or TNF-Rp55$^{-/-}$ bone marrow were prepared from femurs and tibias, filtered, and counted. Female WT or TNF-Rp55$^{-/-}$ mice received a single intravenous injection of 1 × 10^7 bone marrow cells, after being irradiated with 8.5-Gy followed by 4.25-Gy x-rays (MPR-1520R; Hitachi) 4 hours later. The following groups of chimeric mice were generated: WT to WT, WT to TNF-Rp55$^{-/-}$, TNF-Rp55$^{-/-}$ to WT, and TNF-Rp55$^{-/-}$ to TNF-Rp55$^{-/-}$ mice. Genomic DNA was extracted from blood, and bone marrow chimerism was determined 4 weeks later by PCR for the Y chromosome–linked *Sry* gene (forward, 5′-TGGGACTGGTGACAATTGTC-3′; reverse, 5′-GAGTACAGGTGTGCAGCTCT-3′).

Cell transplantation. All animals, including control animals, received daily immunosuppression with cyclosporine A (15 mg/kg, i.p.) initiated 2 days prior to cell transplantation, which was performed under anesthesia with 68.5% N_2O, 30% O_2, and 1.5% isoflurane 24 h after KA injection. Immediately before transplantation, cells were harvested and resuspended at a concentration of 50,000 cells per 2.5 μl in culture medium. Cell injections (50,000 cells per mouse) were performed using a glass capillary (inner diameter of tip, 70–90 μm). The cells were slowly injected in a volume of 2.5 μl using a drill hole above the left hippocampus and a single diagonal injection tract spanning from coordinate (AP, +1.6; ML, +1.2; DV, 0.0) to coordinate (AP, −2.8; ML, −1.75; DV, −4.0), thus depositing the cells within the infrahippocampal cleft of the to-be-injured brain hemisphere as described previously (23). Cells were slowly injected (1 μl/min) while withdrawing the capillary (1 mm/min). The capillary was fully retracted 5 min after injection to avoid reflux of cells. Using an identical procedure, sham-treated control animals (n = 6) received culture medium instead of cells.

Cytokine assay. Eos (1 × 10^6/well) were cultured in RPMI-1640 containing 10% FBS to measure TGF-β1. Supernatants were collected at 24 hours and stored at −80° C until assayed. TGF-β1 proteins released into culture supernatants were assessed using ELISA kits from R&D Systems. The sensitivity of detection was 7 pg/ml.

Cytokine detection. Serum IL-6, IFN-γ, and TNF-α levels were determined using corresponding ELISA kits (R&D Systems). Antibody pairs IL-22 Ab-01 and biotinylated IL-22 Ab-03; IL-17A MAB721 and IL-17A BAF421 (R&D Systems); IL-17F mAb 16-17 and IL-17F 15-1 were used to detect IL-22, IL-17A, and IL-17F, respectively, by sandwich ELISA.

Intracellular cytokine staining. Intracellular cytokine staining was performed on cervical lymph node cells. Cells were restimulated with 50 ng/ml PMA (Sigma-Aldrich), 1 mg/ml ionomycin (Sigma-Aldrich), and GolgiPlug (Pharmingen) for 12 hours. Cells were first stained for CD4 surface antigen and then treated with Cytofix/Cytoperm (Pharmingen) according to the manufacturer's instructions. Intracellular cytokine staining was performed using antibodies to IFN-γ, IL-22, IL-17A, IL-17F, and irrelevant IgG isotype controls. IL-22 Ab-02 antibody was labeled with Alexa Fluor 647 (Molecular Probes), and IL-17F RK015 antibody was labeled with FITC (Pierce Biotechnologies), both according to the manufacturers' instructions. All plots were gated on $CD4^+$ cells, and positive percentages are shown.

Assessment of renal function. Urinary albumin was measured by radial immunodiffusion using a rabbit anti-mouse albumin antibody (Biogenesis) and purified mouse albumin (Sigma-Aldrich) as standards, as previously described (50). Serum urea was measured using a UV method kit (R-Biopharm Rhone) according to the manufacturer's instructions.

Administration of sera containing factor I. For these experiments serum was obtained from mice with combined deficiency of factor H and C3 ($Cfh^{-/-}C3^{-/-}$). An initial intravenous injection of 200 μl of sera was administered to $Cfh^{-/-}Cfi^{-/-}$ animals, and then a further 400 μl of sera was injected intraperitoneally at 24 and 48 hours.

Renal transplant studies. The renal transplantation procedure has been described previously (51). Briefly, the left kidney was removed from 6- to 8-week-old donor mice and preserved in cold saline. The right kidney was removed from recipient mice and the donor kidney transplanted with end-to-side anastomoses of the donor renal vein to the inferior vena cava and the donor aortic cuff to the aorta. Ureter-to-bladder anastomosis was performed to reconstruct the urinary tract. Mice were sacrificed 4–6 weeks after transplant procedure, and renal histology was assessed.

In vivo ECG studies.

- Surface ECG recordings were done under general anesthesia as described (26). ECGs were recorded for 5 minutes at baseline, followed 15 minutes after with intraperitoneal administration of isoproterenol (0.4 mg/kg), and ventricular ectopy was quantified as described (26). Average heart rates after isoproterenol injection were not significantly different among the groups of mice and ranged from 450 to 500 beats per minute, which was significantly lower than those of conscious mice (26), likely due to the effect of general anesthesia.

Patient population

- Patients included in the study met the following criteria: (1) biopsy-proven IMN; (2) creatinine clearance ≥30 ml per min per 1.73 m2; and (3) persistent proteinuria >5 g per 24 h despite treatment with an HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase inhibitor, an ACEi, and/or ARB at maximal tolerated dose for at least 4 months. The Mayo Institutional Review Board and the Research Ethical Board, University Health Network, University of Toronto approved the study protocol. All patients gave written informed consent. Patients who had been on treatment with prednisone, cyclosporine, or mycophenolic mofetil within the last 4 months or alkylating agents within the last 6 months were not included in the study. Patients with active infection, diabetes, or a secondary cause of MN (for example, hepatitis B, systemic lupus erythematosus (SLE), medications, malignancies) were also excluded.

Treatment

- At enrollment, a low-sodium (<4 g day-1) and low-protein (0.8 g per kg per day of high-quality protein) diet was recommended and patients were encouraged to maintain the same diet throughout the duration of the study. All patients received a similar conservative treatment regimen that included loop diuretics to control edema, an HMG-CoA reductase inhibitor, and an ACEi combined with an ARB if tolerated. β-Blockers and non-dihydropyridine calcium channel blockers, in that order, were added when required to control systolic blood pressures to <135 mm Hg in >75% of the readings. Patients who after a minimum of 4 months of conservative therapy and maximized Ang II blockade had proteinuria >5 g per 24 h received two i.v. infusions of rituximab at a dose of 1000 mg on days 1 and 15. To minimize infusion reactions, patients were premedicated with acetaminophen (1000 mg) and diphenhydramine hydrochloride (50 mg) orally. In addition, methylprednisolone (100 mg, i.v.) was given prior to the first rituximab infusion. B-cell depletion was defined as CD19+ count <5 cells per μl at any time and B-cell recovery was defined as CD19+ cell count >15 cells per μl. Patients treated with rituximab, who at month 6 had proteinuria >3 g per 24 h and in whom CD19+ B-cell counts had increased to >15 cells per μl, received a second course of rituximab treatment following the same protocol described above.

Follow-up

- In all patients, clinical and laboratory parameters including complete blood counts, electrolytes, serum albumin, B-cell flow cytometry for CD19+ B cells, serum immunoglobulin (IgG, IgM, IgA) levels, and a lipid panel were evaluated at study entry and at months 1, 3, 6, 9, and 12. Creatinine clearance and protein and creatinine excretion in the urine were assessed by performing two consecutive 24-h urine collections at each time point. Data were considered accurate when urinary creatinine excretion was consistent with a complete 24 h collection. The mean of the two measurements was considered for the analysis. The presence of HACAs was evaluated at baseline and at months 3, 6, 9, and 12.

Method / Approach / Study/ Technique

- A discussion is presented of a problem-solving system
- To improve the efficiency of the method, the following approach may be applied.
- In order to an investigation was made to find the causes of the
- Although large collections of rules and equations have been complied, none are generally accepted
- This approach will be explained and discussed thoroughly in the body of the report.
- This can be accomplished by
- This algorithm to compute the total cost can be described step by step as follows:
- The above preliminary analysis has provided important information
- Various methods have been proposed for selecting an optimum...
- These concepts have been applied to
- On the basis of the concept mentioned above,
- This can be achieved by
- In addition, tissues were stained for infiltrating lymphocytes (CD20 and CD3), and the amount of interstitial fibrosis was quantified by histomorphometry. Formalin-fixed, paraffin-embedded sections were cut onto coated glass slides. Following heat-induced antigen retrieval, sections were incubated at 20°C overnight with either anti-CD20 primary antibody or anti-CD3 primary antibody, both at 1:1000 dilution (Dako, Canada Inc, Mississauga, Ontario, Canada). After rinsing all sections, pretreatment with 3% hydrogen peroxide was performed to prevent endogenous peroxidase activation. Sections were incubated with a secondary rabbit anti-mouse antibody linked with avidin–biotin complex. Sections were counterstained with hematoxylin and examined by light microscopy.
- The HACA assay is a proprietary bridging enzyme-linked immunosorbent assay performed at Genentech Inc. that measures the antibody response to rituximab in human serum samples.
- In all patients, clinical and laboratory parameters including complete blood counts, electrolytes, serum albumin, B-cell flow cytometry for CD19+ B cells, serum immunoglobulin (IgG, IgM, IgA) levels, and a lipid panel were evaluated at study entry and at months 1, 3, 6, 9, and 12.

- This fact suggests that a new concept
- This was accomplished by taking ...
- The preparatory stage is very time consuming process.
- Test are performed for validity, completeness, and compatibility
- There is little hope of achieving successful ...
- There has been an increasing awareness of the potential of using most ..so far made have not taken this approach, with the exception of
- Only a few studies can be found.
- It is a very tedious process to go through
- It is only when .. has been completed that .. may be effected
- The entire interpretation process is conducted in one's head.
- These approaches are sometimes very tedious.
- Several techniques can be used
- A polynomial parametric model can be written as [the following]/[follows]:
- A xx model is constructed/formulated using xx.
- A xx model represents an xx by its xx.
- A process decision model captures the logic essential to
- From the equation above, xx is equal to the summation of xx times the ...
- The validity of a xx model can be checked using Euler's formula.
- Given a model, one can mathematically determine whether ... or ...
- Equations for xx need to be derived and implemented in the system.
- A number of heuristic rules have been developed for
- Optimum .. techniques can be made more reliable by ... so that
- An algorithm based on the characteristic ... is used to determine
- Euler's formula states the following:
- The completed model should agree with the formula.
- For manufacturing purposes, a detailed and precise model of the object is necessary
- Engineering design models are very well defined; therefore,
- To keep the domain narrow enough to be implementable, yet wide enough to be useful.

Point of View

- from an implementation standpoint,
- From the point of view of this application,
- From this point of view, Zadeh suggested an inference rule named xxx (CRI for short).
- Information is the meaningful interpretation and correlation of some aggregation of data in order to allow one to make decisions.
- From a practical point of view, the computational aspects of an FLC require a simplification of the fuzzy control algorithm.
- The use of a hammer to insert screws, although partly effective, tends to distort, destroy, and generally defeat the purpose of using a screw [Kusiak AI Implications for CIM p.129]

Statistical analysis

- Data were analyzed by one-way analysis of variance comparing the three conditions (sham operation, ischemic AKI, and bilateral nephrectomy) at each time point. If significant F-statistic from analysis of variance existed, this test was followed by Dunnett post hoc multiple comparison procedure with sham operation as the control group. For all other comparisons, Student's t-test was used. A P-value of <0.05 was considered as statistically significant.
- Values are expressed as means±s.e.m. and significance was evaluated by Mann–Whitney U test using GraphPad Prism, version 4.0 software (GraphPad Software Inc., San Diego, CA, USA).
- All values are expressed as means±s.d. Statistical significance (defined as P<0.05) was evaluated using analysis of variance and Bonferroni t-tests, and the two-tailed Pearson's test, where appropriate.
- Data are expressed as mean±s.d., median and interquartile range, or frequencies, as appropriate. Variables who deviated from the normal distribution (positively skewed) were log-transformed (log10) before the correlation study.
- Data are represented as means±s.e.m. Student's *t*-test and multiple comparisons with *t*-test *post hoc* analysis of variance were used as indicated below, for the comparison of morphological, immunohistological and functional parameters. Statistical significance was set at $P<0.05$.
- The primary efficacy parameter was defined as change in urinary protein excretion from baseline (week 0) to 12 months after treatment. The 12-month changes were tested against zero using the paired t-test. Secondary end points included 6-month changes in protein; the number with PR or CR at 6 or 12 months; and changes in glomerular filtration rate (GFR), serum albumin, and lipid profiles. Study sample size was based on the desire to have 80% power to detect a drop in urinary protein of at least 2.0 g day-1. Assuming a two-sided hypothesis test performed at a significance level of 0.05 and an s.d. of urinary protein change of 2.5 g, it was determined that 15 patients were required.2 Definition of remission status is according to the criteria established by Cattran et al.30 CR was defined as proteinuria <0.3 g per 24 h, PR as proteinuria ≤3 g per 24 h, and a >50% reduction in peak proteinuria and non-response as <50% reduction in peak proteinuria. Any patient reaching a CR or PR was considered a treatment success
- The statistical significance of differences for the mean values of cytokine concentration and T cell proliferation was determined with Student's *t*-test. Differences with a *P* value of less than 0.05 were considered significant.
- Statistical significance was determined by Student's *t*-test. Data with a *P* value of less than 0.05 were considered significant.
- *In vitro* and *in vivo* experiments were analyzed by the two-tailed Student's *t*-test, with *P* values less than 0.05 considered statistically significant.
- The significance of differences among groups was determined by Student's *t*-test and one-way analysis of variance (SigmaStat software; Jandel).
- The data are presented as mean ± SEM. Differences between the means of the individual groups were assessed by 1-way ANOVA with Duncan’s multiple range tests for the results shown in Figure 5; differences were considered significant at $P < 0.05$. Student’s t

test was used to compare the chow (N) versus L/E groups; significance was determined at $P < 0.05$. The statistical software package Prism 5.0 (GraphPad Software) was used for these analyses.

Comparisons

- As well, the pros and cons of these representations from a process planning point of view will be discussed.
- The method of using xx to implement xx described by Zadeh (1973) appeared more suitable
- As discussed [in the previous section]/[preciously],

Relation

- We can not invert F' directly because it defines a many-to-one mapping.
- The relationships appear very complicate
- Lifting tasks involve complex and imprecise relationship between the task variables and the human operator's characteristics.
- These methods are based on the relationship between ... and ...
- The fundamental concept of a fuzzy rating language is that we can establish a relationship among terms such as high, medium, and low, and then modify these relationships.
- This article will thus mention the latter as well as the former.
- The former two bear a close relation to a fuzzy Cartesian product.

Importance

- The emphasis is on an implementation of a general approach to rule based decision making.

Consideration / Attention

- Careful evaluation is necessary to ensure
- Such a formulation does not change further considerations.
- Considerable attention has been paid to
- Attention should be paid to an important finding of this investigation.
- Caution should be exercised in this process to avoid ...
- Primary consideration is given to ... components, though others can be accommodated
- After ... has been defined by ..., a carefully analysis is carried out/performed to determine
- A number of factors such as ...need to be taken into consideration before making the appropriate decision.
- It should be noted that
- It is important to point out that ...
- These considerations have heightened interest in the possibility of providing ...
- We should stress the fundamental importance of the xx

Results.

Advantages / Disadvantage

- One of the major advantages of this new measure of xx is that it can be applied to the experimental study of
- One advantage of using a .. is the ease of preparing it.
- It has a very fast decision making process
- All the algorithms involve mostly logical operations.
- It can be easily and without additional cost implemented in a microprocessor;based environment.
- It can reduce the waste of designing from scratch.
- The advantages of using a xx to represent xx are the following:
- However, xx is not without its shortcomings.
- In most cases, the xxx shows an improvement over the existing xxx.
- Compared to the existing xx, the impacts of the xx are generally reduced by 5% to 9%.
- The "best case" results shows a savings of 6% to 9%.
- Most of the existing works based on xx approach can only recognize a xx .
- Most of the above methods are computational expansive and limited to xx.
- Some other advantages of xx are the following:
- The problem is the limitation of this method to a limited domain of parts.
- It proved limited in application because it demanded precision in system modeling that was impossible in practice.
- There are advantages to be gained in the structuring of costs and benefits, the use of xx,
- The disadvantages of this method are also disadvantages of conventional xx approaches.
- This combines the best features of both techniques
- Hopefully, this tool can be as the reference framework of for developing a xx platform, and helping the administration, marketing, and knowledge management activities in virtual communities.

Results

- An improvement on the result shown above can be made by based on the data provided
- Recent work has identified
- Time-dependent changes of
- Cyclosporine-induced cell death is triggered by a non-classical phosphoinositide 3-kinase and does not require ERK activation.
- Much of the current work on
- Discussion of these theories is beyond the scope of this review
- Based on the information contained in this
- The result can be categorized into nine classes
- The results are illustrated by an example
- The experimental results for each xx time are reported in Table 2.
- From the results obtained so far, it seem that
- Because of the inaccuracy of the ..., a conclusion cannot be drawn as
- Although much effort has been made to., this reality is far from completion.
- The results indicate that the total benefits are higher than the total costs.

- Their results may then serve as guidelines for lower level models, less fuzzy and more detailed.

Conclusion

- From the discussion, one may conclude that ...
- The first indication that
- In summary, we have shown that
- These results suggested that
- Our findings have shown that
- Our observations have provided
- This study reveals following five main findings:
- To our knowledge, this is the first immunohistochemical report of both PIM and HIFs in the diabetic kidney.
- As mentioned above,
- Moreover, we demonstrate that,
- A number of studies have elaborated a body of knowledge about
- There are findings to indicate that
- On a descriptive level, there is agreement that for
- One issue that became particularly evident from our study is
- Our data are in agreement with the findings and models presented by previous publications, in particular those of Jones *et al*
- According to our observations,
- …was not evident from our data
- It could therefore be concluded that
- our data illustrate that
- All these different aspects, as listed above, lead to
- In fact, it has already been speculated that
- It should be furthermore mentioned in this context that
- To identify and verify the essential factors triggering developmental renin expression, it is of interest now to study the development of intrarenal renin expression in mice with defined gene mutations, using the results of this study as a reference system.
- In conclusion, using several approaches, we demonstrated that
- But we have not provided formal proof yet that
- In summary, our results suggest that
- In conclusion, we identify
- A better understanding of such regulatory systems may yield novel therapeutic approaches to glomerular diseases.
- Preliminary results of short-term studies (2 weeks) indicate that
- However, we believe that
- However, despite our extensive measurements of these parameters, we found no relationship between response and initial proteinuria, time to B-cell recovery, the development of antibodies to rituximab, B-cell count in the kidney tissue, nor the degree of tubular interstitial damage present. A clear conclusion for efficacy, however, cannot be

obtained from an uncontrolled study, and definitive claims of efficacy should be reserved for rigorous, prospective, controlled, and randomized trials with the use of rituximab and that will also look for factors that may determine its efficacy in IMN.

- It has become increasingly clear that
- Form the above discussion, the conclusion can be reached that
- The conclusions drawn are also valid
- In conclusion to this, it becomes obvious that the problem of xx lies not only in...
- We have attempted to introduce some concepts associated with a theory of
- Considerable more work, hopefully, will be done in this area
- Employing the compositional rule of inference, the assessment of the xx compatibility in achieving prescribed xx projectiles in any level of the hierarchy is made possible.
- This paper has presented a theoretical and experimental study of the xx process and xx concept.
- The experimental research results will hopefully serve as useful feedback information for improvements for xx work.
- The scope of this contribution was to introduce a xx method.
- This has a tremendous impact on our understanding of
- Significant progress has been made in advancing RNAi therapeutics in a remarkably short period of time
- To date, little is known about…
- Irrespective of the mechanisms and the molecules –which are still largely unknown – involved in MSC functions, current data suggest that
- To better understand

Future Research (常用于 conclustion 的结尾)

- Thus, first extension of the approach could be,
- Further studies are needed to determine the importance of
- Some improvements to the scheduling aspect of the model may be brought through additional levels in the hierarchy for more detailed representation of the scheduling activity.
- It also unveiled new aspects of the role played by thyroid hormone in response to injury and tissue repair.
- In the near future, the ongoing clinical trials with siRNAs for macular degeneration and RSV may reveal the exciting potential of RNAi therapeutics as the next major class of drug molecules.
- In the next few years it should become clear whether
- It should be an exciting time for researchers seeking to harness this powerful endogenous pathway to treat human disease.

Acknowledgement

- The authors are grateful to the insightful work provided by Mr. John W. Harney and all of the fellows and students that contributed with some of the studies discussed in this article

- I would like to express my gratitude to all those who gave me the possibility to complete this thesis.
- I want to thank the Department of Transport of the former Interim Government of Namibia for giving me permission to commence this thesis in the first instance, to do the necessary research work and to use departmental data
- This work was supported by NIH grants AI058695 and AI056900. We thank members of the laboratory and our collaborators for many useful suggestions.
- We thank J. Maraganore and B. Greene for critical reading of the manuscript, M. Duckman for graphics assistance and A. Capobianco for word processing assistance.

Author contributions

- All authors were involved in experimental planning and data analysis and contributed to manuscript preparation.

Tables and Figures

- Figure 7-1 sketches these relationships.
- The graphical representation of these functions is shown in Figure 1.
- The xx may be depicted as in Figure 1.
- Figure x shows the schematic diagram of the
- Figure 1 though 2 provide a ... that
- the architecture of this expert system for .... is illustrated in Figure 2.
- Figure 2 gives the outline of an ... system
- Table shows the
- Time-dependent changes of
- as shown in Table 1 and 2
- This concept is illustrated in Figure 2
- At the top of Table xx are shown two blocks of data.
- Each table or matrix has constructs xx through xx as row;headings,
- xx through xx as column;headings.
- Time course of calculated renin immunoreactive tissue volumes during development of the mouse kidney. Data are single values per time point. They were derived from the kidneys shown in Figure 1.
- Yellow color indicates
- PCT3 cells were pretreated with 50 μM PD98059 or 10 μM U0126 for 30 min and then cells were stimulated with 25 μg ml^{-1} CsA for 6 h. ERK and PKB activation were analyzed by western blot using phospho-specific antibodies.
- ERK and PKB activation were measured by western blot using phospho-specific antibodies
- The data are the means of three different experiments taking the number of untreated cells (vehicle alone) as 100%.
- ERK and PKB activation were then analyzed by western blot with the corresponding phospho-specific antibodies. To ensure equal amount of protein in each lane, western blots against total protein were performed for ERK and PKB.

- The means of optical density (OD) values of the bands detected in western blots of three different experiments are plotted, taking the maximal value of phosphorylation of ERK and PKB as 100%.
- CT3 cells were treated with 25 μg ml^{-1} CsA or vehicle alone for 24 h and then fixed with paraformaldehyde and stained with Hoescht as indicated in Materials and Methods. Then cells were visualized by phase-contrast microscopy (upper panel) and fluorescence microscopy (lower panel).
- The data are the means of three experiments performed on different days taking the number of cells at the onset of the experiment as 100%.
- Rats were killed at 4 h and at days 1, 2, 4, 7, 9, 14, 21, and 28 after disease induction (n=9 each).
- $^{*}P$<0.05 versus non-nephritic rats.
- The expression of the CCN3 protein was detected by western blot analysis (n=4 each, PC, positive control, M, molecular weight markers).
- Original magnifications: ×200 in A, E, G, H; ×600 in B, C, D, F.
- Data are means±s.d. of four independent experiments.
- $^{*}P$<0.05 versus 0.5 h.
- PDGF-BB and -DD, but not PDGF-AA and -CC, induce a downregulation of *CCN3* mRNA as determined by real-time RT-PCR and normalized to *glyceraldehyde-3-phosphate dehydrogenase* mRNA.
- Cell number was counted in duplicate using a Malassez hemocytometer
- Data are means±s.d. of four independent experiments. $^{*}P$<0.05 versus cells treated with MCDB media for 24 h, #P<0.05.
- The curves are adjusted for history of coronary artery disease, CRP, and progression to ESRD.
- Both models are independent of age, gender, baseline GFR and proteinuria, and other clinical characteristics and traditional and nontraditional cardiovascular risk factors
- Correlation between serum bone-specific alkaline phosphatase (bAP) and serum PTH (n=99, P<0.0001, r^2=0.190)
- Concentration of serum FGF23 concentration before starting and at the end of the 4-h hemodialysis procedure in 23 patients, expressed as $\log_{10}$ values (n=23, P<0.0001, Student's paired t-test).
- Immunohistochemistry for the hypoxia marker pimonidazole (PIM) and HIF-2α; arrow=endothelial cell and CD=collecting duct
- Magnification: ×1000.
- A table of .. is developed and significant recommendations are made.

CONJUGATION

To Indicate Addition

- additionally, again, also, and then, as can be easily understood, besides, equally important, especially, finally, for the same reason, first, further, furthermore, in addition, last,

likewise, moreover, next, second, third, too, evidently, obviously, roughly speaking, broadly speaking

To Indicate Cause and Effect

- accordingly, as a result, consequently, for this reason, hence, in short, otherwise, then, therefore, thus, truly

To indicate Comparison

- in a like manner, likewise, similarly , alternatively

To Indicate Concession

- after all, although this may be true, at the same time, even though, even so , I admit, naturally, of course

To Indicate Contrast

- and yet, at the same time, but, for all that, however, yet, in fact, in contrast, in the real life, in spite of, nevertheless, notwithstanding, normally, on the contrary, on the other hand, still, traditionally, rather, unfortunately,

To Indicate Time Relationships

- after a short time, afterwards, as indicated earlier, as long as, as soon as, at last, at length, at the moment, at that time, at the same time, before, earlier, currently, immediately, in the meantime, in recent years, lately, later, meanwhile, often, of late, presently, recently, soon, shortly, since, thereupon, temporarily, therefore, until, when, while

To Indicate Special Features or Examples

- for example, for instance, incidentally, indeed, in fact, in other words, in particular, in practice, specifically, that is, to illustrate, in this respect, theoretically, as mentioned before / above

To Indicate Summary

- in brief, in conclusion, in short, in summary, on the whole, to conclude
- , in general, to summarize, to sum up, as a result, ultimately,

VERB PHASE

- build a .. model
- have been described to
- To assess the functional role of
- we detected
- build up the key link
- Although a number of studies have identified predictors of
- began a new era in ...
- can be regarded as / achieved / used to/for / found / obtained through

- can result in
- carries out ... tasks
- production information in order to simultaneously
- contains all information necessary to describe
- do not make use of production information
- deals with
- end with failure
- fetch the information from the model directly
- has great potential / yet to be resolved / spurred the development of /
- been recognized as
- site component / cable of / currently implemented for / demonstrated by an example / finally reached / made equal to / equivalent to / more suitable to / oriented to / interpreted as / pointed out / potentially of great benefit in the complex task of / shown in / used to effectively guide the search
- makes use of
- was significantly higher as compared to
- To assess disease-specific alterations, we measured
- To specifically assess the relationship between
- make up
- To determine if
- This statistically significant difference still
- They positively correlated with
- Present
- Our results are consistent with our recent data that suggest that
- contribute to
- meets the needs of real life production,/ the current demands placed upon it
- must be justified
- point out
- play an important role
- relates to
- rely on
- We performed
- satisfy the needs
- determine the total requirements for the ...
- uses ... as a key to search for...
- without relying on
- will be available/ performed/ overlooked

NOUN PHASE

- a basic technical function of
- a significant reduction of
- drug variability
- a critical need

- negatively correlated with
- a key / principle feature of
- a substantial impact on
- an intensive review was conducted
- The analysis of the correlations by
- an increasing need for expanding the application of ....
- an important component / function / aspect / issue
- each rule is numbered in sequence
- each of these involves
- for this calculation, it is necessary to define
- in the physical environment / integration of
- in the reality of situations where …
- many aspects of
- most past efforts have been spent on ...
- common sense to a well studied and documented technical field.
- sources of additional information on ... are listed
- systematic and rationally structured format
- the basis on which a range of ...operations can be established is shown
- THE basic philosophy / principles of / key element / general hypothesis / candidate list of / concept of ... has attracted wide interest / function is concerned with / heart / impact / nature / role / task of / kernel functions
- the number of parts needed to
- the above statement means that
- the output data is passed to
- the proposed method / underlying principle
- the recommendations made in this report, if implemented, should
- this information resides in
- this process is composed of ... different ... operation
- along with the use of
- concerning general aspects of
- due to
- for later use in generating...,
- in turn,
- it can be claimed/concluded that
- it demonstrates the decisions required of
- it also provides information to ..
- it becomes essential to
- let ... be the probability that
- once... is written, it is compiled into...
- suppose it is observed that
- this is because
- this results in a
- upon completion of the ... analysis,

- when the knowledge is of mathematics or quantum physics, it will also be
- recorded in books and papers
- selection of rules for using the tools, for generating operation plans,
- is another matter of preference, since practice varies greatly.
- for the sake of convenience
- correct decision to be reach
- keeping the number of rules to a minimum.
- a good process plan will result exhibiting several characteristics:
- practical solutions
- because of rather small job lot sizes
- Backward reasoning can be used to answer the question "should milling tool be select" different level of knowledge in the realm of process planning